(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2012 (23.08.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/112902 A1

(51) **International Patent Classification**:
A01N 37/00 (2006.01) ***A61K 31/20*** (2006.01)
C12P 7/64 (2006.01)

(21) **International Application Number**:
PCT/US2012/025666

(22) **International Filing Date**:
17 February 2012 (17.02.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/444,696 18 February 2011 (18.02.2011) US

(71) **Applicant** *(for all designated States except US)*: **MARTEK BIOSCIENCES CORPORATION** [US/US]; 6480 Dobbin Road, Columbia, MD 21045 (US).

(72) **Inventor; and**
(75) **Inventor/Applicant** *(for US only)*: **TANG, Xuejun** [US/US]; 3506 Lowlen Court, Ellicott City, MD 21042 (US).

(74) **Agents**: **KIM, Judith, U.** et al.; Sterne, Kessler, Goldstein & Fox PLLC, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2012/112902 A1

(54) **Title**: METHODS OF PREPARING FREE POLYUNSATURATED FATTY ACIDS

(57) **Abstract**: Provided herein are methods of making compositions of free fatty acids and alkyl esters of polyunsaturated fatty acids (PUFAs). Also provided are compositions comprising a PUFA selected from a free fatty acid or a ester wherein the PUFA is made according to a method of the invention.

METHODS OF PREPARING FREE POLYUNSATURATED FATTY ACIDS

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] Provided herein are methods of making compositions of free fatty acids and alkyl esters of polyunsaturated fatty acids (PUFAs). Also provided are compositions comprising a PUFA selected from a free fatty acid or a ester wherein the PUFA is made according to a method of the invention.

Background

[0002] Methods of making free fatty acids and ethyl esters have been reported, for example in U.S. Patent Number 6,486,942. Free fatty acids can be used to make esters of polyunsaturated fatty acids. Uses of esters and free fatty acids of polyunsaturated fatty acids in emulsions to treat, for example liver disease and other disorders as reported in WO 2011/103510 and WO 2011/103512.

BRIEF SUMMARY OF THE INVENTION

[0003] Provided herein is a process for making a free fatty acid of a polyunsaturated fatty acid (PUFA) comprising:

(a) adding acetone to the salt solution to form a precipitate wherein the acetone is added before any significant acidifying step is performed;

(b) separating the precipitate from the solution; and

(c) acidifying the solution to produce a free fatty acid PUFA.

[0004] Also provided herein is a process for making a free fatty acid of a polyunsaturated fatty acid (PUFA) comprising:

(a) saponifying a PUFA, selected from a glyceride or an ester, to form a PUFA salt solution;

(b) adding acetone to the salt solution to form a precipitate wherein the acetone is added before any significant acidifying step is performed;

(c) separating the precipitate from the solution; and

(d) acidifying the solution to produce a free fatty acid PUFA.

DETAILED DESCRIPTION OF THE INVENTION

[0005] For the descriptions herein and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a compound" refers to more than one compound.

[0006] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0007] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0008] In reference to the present disclosure, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise.

[0009] Also provided herein is a process for making a free fatty acid of a polyunsaturated fatty acid (PUFA) comprising:

a) saponifying a PUFA triglyceride to form a PUFA salt solution;

b) adding acetone to the salt solution to form a precipitate wherein the acetone is added before any significant acidifying step is performed;

c) filtering the precipitate to form a filtrate; and

d) acidifying the filtrate to produce a free fatty acid PUFA.

[0010] In some embodiments the saponification step is carried out on a PUFA glyceride or ester, more particularly on a triglyceride, using a base. In some embodiments the saponification step is carried out with an aqueous alkali. In one embodiment, the aqueous alkali is selected from the group consisting of potassium hydroxide and sodium hydroxide, more particularly sodium hydroxide. In some embodiments the saponification step further comprises addition of a water miscible solvent such as an alcohol or tetrahydrofuran, more particularly an alcohol selected from methanol, ethanol, propanol, isopropanol, and butanol (also isobutyl, sec-butyl and *tert*-butyl alcohol), and more particularly ethanol.

[0011] In some embodiments, the saponification step or hydrolysis step is carried out by mixing the aqueous alkali with the alcohol for a time and temperature sufficient to

convert the PUFA glycerides or esters, in particular triglycerides, to a PUFA salt. In a particular embodiment, the mixture is heated to about 50 °C for about three hours.

[0012] In some embodiments, unsaponified materials may be removed by extraction with, for example an organic nonpolar solvent such as, but not limited to, methylene chloride, petroleum either, or ethyl ether. In a further embodiment, a process provided herein is carried out in the absence of a step to remove unsaponified material with an organic non-polar solvent.

[0013] In the precipitation step, acetone is added to the salt solution to form a precipitate. In some embodiments the acetone is added to the salt solution and stirred for example, but not limited to, from about 2 to about 16 hours. In some embodiments, the solution is allowed to precipitate at temperature from about 25 °C down to about -20 °C, in particular from about 10 °C down to about -20 °C, more particularly from about 0 °C down to about -20 °C. In some embodiments, a particular ratio of acetone to $EtOH/H_2O$ is about 7 to 1. In some embodiments the acetone is stirred for example at a temperature in the range of from about -20 °C to about room temperature (25 °C). The precipitation step is carried in the substantial absence of any acidifying step. In a particular embodiment an acidifying step is performed after the precipitation step. In a particular embodiment, the precipitation step is carried in the absence of an acidifying step (e.g., no added acid). In some embodiments, a process is provided herein wherein the acetone is added to the salt solution to form a precipitate wherein the acetone is added before removing unsaponified materials.

[0014] In some embodiments, the precipitate is separated from the solution. The separation of the precipitate can be carried out using methods typically used to separate precipitates from liquid solutions. Non-limiting examples of such methods include filtration and centrifugation. The remaining solution or filtrate is evaporated. In a particular embodiment, the precipitate is filtered off and the filtrate evaporated and a concentrated aqueous mixture is collected. In a particular embodiment, the solution or filtrate is evaporated *in vacuo*. In further embodiment, the solution or filtrate is evaporated at a temperature from about 25 °C to about 50 °C. More particularly, the solution or filtrate is evaporated at a temperature at about 25 °C. In some embodiments the solution or filtrate is evaporated to about 1/6 of the volume of the solution prior to evaporation.

[0015] In some embodiments the resulting solution, in particular embodiments the filtrate, more particularly the solution or filtrate after evaporation is acidified to produce free fatty acids of the PUFAs. In some embodiments, the resulting solution or filtrate after evaporation is diluted with water particularly to about 1.5 ml/g oil. The acidification step may be carried out with for example, but not limited to, mineral or organic acid sufficient to convert the PUFA salt to the corresponding free fatty acid. In a particular embodiment, the acid is selected from group consisting of hydrochloric acid, sulfuric acid, phosphoric acid, nitric acid, potassium hydrogen sulfate, sodium hydrogen sulfate, and citric acid.

[0016] In some embodiments, the resulting aqueous mixture (after the acidification step) is stirred or agitated to ensure complete conversion of the PUFA salt to free fatty acid. In some embodiments, the resulting aqueous mixture is stirred up to about 2 hours. In another embodiment, the resulting aqueous mixture is stirred for at least about 2 hours. In a further embodiment, the resulting aqueous mixture is stirred or agitated at room temperature.

[0017] The free fatty acids may be obtained from the resulting solution, more particularly from the filtrate, via solvent extraction or without solvent extraction. Any solvent that is suitable for extracting PUFAs from solutions may be used, particularly an organic solvent. In one particular embodiment the PUFA free fatty acids are extracted at least once with a hexane. In some embodiments, the hexane extract is washed, for example with saline and then filtered. The filtrate is evaporated to obtain crude PUFA free fatty acid oil.

[0018] In some embodiments, the PUFA free fatty acids are obtained from the resulting solution without a solvent extraction step. In particular, the resulting solution or filtrate containing the PUFA free fatty acids is allowed to form an oil layer which is then split from the solution and evaporated. In some embodiments, the oil layer is evaporated overnight under vacuum.

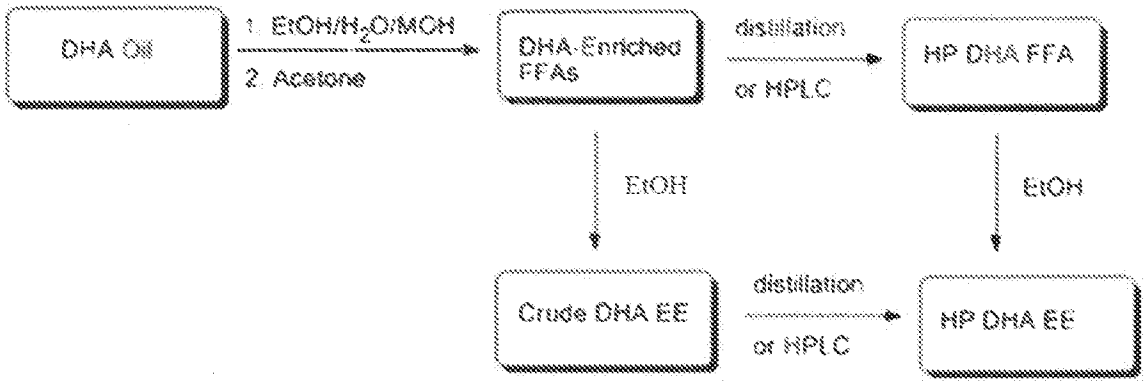
[0019] The resulting free fatty acids may be converted to alkyl esters, and in particularly embodiments, the free fatty acids are converted to an ester selected from methyl or ethyl esters using methods known to skilled artisans.

[0020] In some embodiments provided herein, the PUFA free fatty acids are highly enriched and provided in a pure form.

[0021] In particular embodiments, the PUFA free fatty acids may be purified by methods known by skilled artisans such as through distillation or High Pressure Liquid Chromatography.

[0022] In some embodiments, the free fatty acid product comprises DHA.

[0023] In some embodiments, the alkyl esters, particularly ethyl esters, can be made from DHA-enriched or high potency (HP) PUFA free fatty acids, according to the following scheme(s):



MOH = metal hydroxide

[0024] Some embodiments provided herein allow for large-scale production to achieve highly-enriched long-chain polyunsaturated free fatty acids and their ester forms in the absence of an additional step selected from an acidifying step, a salt formation step, a precipitation step, a urea adduction step, and a solvent extraction step.

[0025] Some embodiments provided herein allow for large-scale production to achieve highly-enriched long-chain polyunsaturated free fatty acids and their ester form without: 1) multiple acidifying steps; 2) multiple salt formation steps; 3) multiple precipitation steps; 4) urea adduction step; and/or (5) solvent extraction for the recovery of free fatty acids.

[0026] Some embodiments provided herein also provide an efficient process to achieve polyunsaturated fatty acid esters, free fatty acids, and triglycerides in high purity without lengthy distillation cycles.

[0027] Any source of polyunsaturated fatty acids, including but not limited to, sources of arachidonic acid (ARA), docosahexaenoic acid (DHA), docosapenaenoic acid (DPAn-6), and eicosapentaenoic acid (EPA) can be used in the compositions and methods described herein, including, for example, animal, plant and microbial sources. In some embodiments, a source of oils containing PUFAs, including but not limited to sources of

ARA, DHA, DPAn-6 and EPA suitable for the compositions and methods described herein is an animal source. Examples of animal sources include aquatic animals (e.g., fish, marine mammals; crustaceans such as krill and other euphausids; rotifers, etc.) and lipids extracted from animal tissues (e.g., brain, liver, eyes, etc.) and animal products such as eggs or milk. Examples of plant sources include macroalgae, flaxseeds, rapeseeds, corn, evening primrose, soy and borage. Examples of microorganisms include microalgae, protists, bacteria and fungi (including yeast). For example, the PUFAs, including but not limited to ARA, DHA, DPAn-6, and EPA may be purified from fish oil, plant oil, seed oil, or other naturally occurring oils such that the DHA to EPA ratio are within the scope described herein.

[0028] In some embodiments, the composition of DHA is a microbial oil or is derived from microbial oil. Exemplary microbes, from which microbial oil may be obtained, include, among others, the microbial groups Stramenopiles, Thraustochytrids, and Labrinthulids. Stramenopiles includes microalgae and algae-like microorganisms, including the following groups of microorganisms: Hamatores, Proteromonads, Opalines, Develpayella, Diplophrys, Labrinthulids, Thraustochytrids, Biosecids, Oomycetes, Hypochytridiomycetes, Commation, Reticulosphaera, Pelagomonas, Pelagococcus, Ollicola, Aureococcus, Parmales, Diatoms, Xanthophytes, Phaeophytes (brown algae), Eustigmatophytes, Raphidophytes, Synurids, Axodines (including Rhizochromulinaales, Pedinellales, Dictyochales), Chrysomeridales, Sarcinochrysidales, Hydrurales, Hibberdiales, and Chromulinales. The Thraustochytrids include the genera *Schizochytrium* (species include *aggregatum, limnaceum, mangrovei, minutum, octosporum), Thraustochytrium* (species include *arudimentale, aureum, benthicola, globosum, kinnei, motivum, multirudimentale, pachydermum, proliferum, roseum, striatum), Ulkenia* (species include *amoeboidea, kerguelensis, minuta, profunda, radiate, sailens, sarkariana, schizochytrops, visurgensis, yorkensis), Aplanochytrium* (species include *haliotidis, kerguelensis, profunda, stocchinoi), Japonochytrium (species* include *marinum), Althornia* (species include *crouchii),* and *Elina* (species include *marisalba, sinorifica).* The Labrinthulids include the genera *Labyrinthula* (species include *algeriensis, coenocystis, chattonii, macrocystis, macrocystis atlantica, macrocystis macrocystis, marina, minuta, roscoffensis, valkanovii, vitellina, vitellina pacifica, vitellina vitellina, zopfi), Labyrinthomyxa* (species include *marina), Labyrinthuloides*

(species include *haliotidis, yorkensis), Diplophrys* (species include *archeri), Pyrrhosorus** (species include *marinus), Sorodiplophrys** (species include *stercorea),* and *Chlamydomyxa** (species include *labyrinthuloides, montana)* (* = there is no current general consensus on the exact taxonomic placement of these genera).

[0029] In some embodiments, the microbial oil source is oleaginous microorganisms, such as certain marine algae. As used herein, "oleaginous microorganisms" are defined as microorganisms capable of accumulating greater than 20% of the dry weight of their cells in the form of lipids. In some embodiments, the DHA is derived from a phototrophic or heterotrophic single cell organism or multicellular organism, e.g., an algae. For example, the DHA may be derived from a diatom, e.g., a marine dinoflagellates (algae), such as *Crypthecodinium sp., Thraustochytrium sp., Schizochytrium sp.*, or combinations thereof. Exemplary samples of *C. cohnii*, have been deposited with the American Type Culture Collection at Rockville, MD, and assigned the accession numbers 40750, 30021, 30334-30348, 3054130543, 30555-30557, 30571, 30572, 30772-30775, 30812, 40750, 50050-50060, and 50297-50300.

[0030] As used herein, the term microorganism, or any specific type of organism, includes wild strains, mutants or recombinant types. Organisms which can produce an enhanced level of oil containing DHA are considered to be within the scope of this invention. For example, cultivation of dinoflagellates such as *C. cohnii* has been described previously. *See*, e.g., U.S. Pat. No. 5,492,938 and Henderson *et al.*, *Phytochemistry 27*:1679-1683 (1988). Also included are microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of DHA as the comparable wild-type strains.

[0031] Organisms useful in the production of DHA can also include any manner of transgenic or other genetically modified organisms, such as a genetically modified plant or a genetically modified microorganism manipulated to produce DHA. e.g., plants, grown either in culture fermentation or in crop plants, e.g., cereals such as maize, barley, wheat, rice, sorghum, pearl millet, corn, rye and oats; or beans, soybeans, peppers, lettuce, peas, Brassica species (e.g., cabbage, broccoli, cauliflower, brussel sprouts, rapeseed, and radish), carrot, beets, eggplant, spinach, cucumber, squash, melons, cantaloupe, sunflowers, safflower, canola, flax, peanut, mustard, rapeseed, chickpea, lentil, white clover, olive, palm, borage, evening primrose, linseed, and tobacco. In some

embodiments, the DHA is derived from a soybean source, including wild type and genetically modified soybean sources.

[0032] In some embodiments, the DHA may be purified in the form of free fatty acids, fatty acid esters, phospholipids, triglycerides, diglycerides, monoglycerides or combinations thereof by any means known to those of skill in the art. In some embodiments, the DHA comprises an ester. The term "ester" refers to the replacement of the hydrogen in the carboxylic acid group of the DHA molecule with another substituent. Typical esters are known to those in the art, a discussion of which is provided by Higuchi, T. and V. Stella in "Pro-drugs as Novel Delivery Systems," Vol. 14, A.C.S. Symposium Series, Bioreversible Carriers in Drug Design, Ed. Edward B. Roche, American Pharmaceutical Association, Pergamon Press, 1987, and Protective Groups in Organic Chemistry, McOmie ed., Plenum Press, New York, 1973. In some embodiments, the ester is an alkyl ester. Examples of more common esters include C_1-C_6 esters, e.g., methyl, ethyl, propyl, butyl, pentyl, hexyl, or branched variations thereof, e.g., isopropyl, isobutyl, isopentyl, or t-butyl. In some embodiments, the ester is a carboxylic acid protective ester group, esters with aralkyl (e.g., benzyl, phenethyl), esters with lower alkenyl (e.g., allyl, 2-butenyl), esters with lower-alkoxy-lower-alkyl (e.g., methoxymethyl, 2-methoxyethyl, 2-ethoxyethyl), esters with lower-alkanoyloxy-lower-alkyl (e.g., acetoxymethyl, pivaloyloxymethyl, 1-pivaloyloxyethyl), esters with lower-alkoxycarbonyl-lower-alkyl (e.g., methoxycarbonylmethyl, isopropoxycarbonylmethyl), esters with carboxy-lower alkyl (e.g., carboxymethyl), esters with lower-alkoxycarbonyloxy-lower-alkyl (e.g., 1-(ethoxycarbonyloxy)ethyl, 1-(cyclohexyloxycarbonyloxy)ethyl), esters with carbamoyloxy-lower alkyl (e.g., carbamoyloxymethyl), and the like. In some embodiments, the added substituent is a cyclic hydrocarbon group, e.g., C_1-C_6 cycloalkyl, or C_1-C_6 aryl ester. Other esters include nitrobenzyl, methoxybenzyl, benzhydryl, and trichloroethyl. In some embodiments, the ester substituent is added to a DHA free acid molecule when the DHA is in a purified or semi-purified state. Alternatively, the DHA ester is formed upon conversion of a triglyceride to an ester. One of skill in the art can appreciate that some non-esterified DHA molecules can be present in the DHA compositions, e.g., DHA molecules that have not been esterified, or DHA triglyceride ester linkages that have been cleaved, e.g., hydrolyzed. In some embodiments, the non-esterified DHA molecules or the DHA

triglyceride molecules constitute less than 3% (mol/mol), about 0.01% to about 2% (mol/mol), about 0.05% to about 1% (mol/mol), or about 0.01% to about 0.5% (mol/mol) of the total DHA molecules. In some embodiments, the amount of ethyl ester of DHA in the compositions may be at least about 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt%.

[0033] In some embodiments, the DHA of the present invention is a triglyceride, diglyceride or monoglyceride. A "triglyceride" is a glyceride in which the glycerol is esterified with three fatty acid groups. Typical triglycerides are known to those in the art. In some embodiments, the DHA is in the form of a triglyceride or a diglyceride, wherein one or more fatty acid groups other than DHA are present in the triglyceride or diglyceride. In some embodiments, DHA is the only fatty acid group on a triglyceride or diglyceride molecule. In some embodiments, one or more fatty acid groups of a triglyceride have been hydrolyzed or cleaved.

[0034] In some embodiments, the DHA of the present invention is in the form of free fatty acid. "Free fatty acid" refers to fatty acid compounds in their acidic state, and salt derivatives thereof.

[0035] In the embodiments described herein, the composition of DHA for use in the methods may be obtained by standard techniques known in the art. In some embodiments, EPA may be removed during the purification of DHA, or alternatively, the DHA may be from an organism that produces DHA with the levels of EPA described herein, for example a production organism is selected that produces DHA with an insubstantial amount of EPA. DHA can be purified to various levels. DHA purification can be achieved by any means known to those of skill in the art, and can include the extraction of total oil from an organism which produces DHA. In some embodiments, EPA, ARA, and/or DPAn6 are then removed from the total oil, for example, via chromatographic methods. Alternatively, DHA purification can be achieved by extraction of total oil from an organism which produces DHA, but produces little, if any, EPA, ARA, DPAn6, and/or flavonoids. In some embodiments, the oil can be diluted with other oils, such as sunflower oil, to achieve the desired concentration of fatty acids.

[0036] Microbial oils useful in the present invention can be recovered from microbial sources by any suitable means known to those in the art. For example, the oils can be recovered by extraction with solvents such as chloroform, hexane, methylene chloride, methanol and the like, or by supercritical fluid extraction. Alternatively, the oils can be

extracted using extraction techniques, such as are described in U.S. Pat. No. 6,750,048 and International Pub. No. WO 2001/053512, both filed Jan. 19, 2001, and entitled "Solventless extraction process," both of which are incorporated herein by reference in their entirety. Processes for the preparation of various forms of DHA are also described in, among others, US Patent Publication No. 2009/0023808 "Production and Purification of Esters of Polyunsaturated Fatty Acids" by Raman et al., and US Patent Publication No. 2007/0032548 "Polyunsaturated fatty acids for treatment of dementia and pre-dementia-related conditions" by Ellis, incorporated herein by reference.

[0037] Additional extraction and/or purification techniques are taught in International Pub. No. WO 2001/076715; International Pub. No. WO 2001/076385; U.S. Pub. No. 2007/0004678; U.S. Pub. No. 2005/0129739; U.S. Pat. No. 6,399,803; and International Pub. No. WO 2001/051598; all of which are incorporated herein by reference in their entirety. The extracted oils can be evaporated under reduced pressure to produce a sample of concentrated oil material. Processes for the enzyme treatment of biomass for the recovery of lipids are disclosed in International Pub. No. WO 2003/092628; U.S. Pub. No. 2005/0170479; EP Pat. Pub. 0776356 and U.S. Pat. No. 5,928,696, the last two entitled "Process for extracting native products which are not water-soluble from native substance mixtures by centrifugal force," all of which are incorporated herein by reference in their entirety.

[0038] Methods of determining purity levels of fatty acids are known in the art, and may include, e.g., chromatographic methods such as, e.g., HPLC silver ion chromatographic columns. Alternatively, purity levels may be determined by gas chromatography, with or without converting DHA to the corresponding alkyl ester. The percentage of fatty acids may also be determined using Fatty Acid Methyl Ester (FAME) analysis.

[0039] In some embodiments, the DHA esters can be derived from undiluted oil from a single cell microorganism, and in some embodiments, from undiluted DHASCO-T (Martek Biosciences Corporation, Columbia, MD). In some embodiments, the oil from which DHA compositions can be derived includes single cell microorganism oils that are manufactured by a controlled fermentation process followed by oil extraction and purification using methods common to the vegetable oil industry. In certain embodiments, the oil extraction and purification steps can include refining, bleaching, and deodorizing. In some embodiments, the undiluted DHA oil comprises about 40% to

about 50% DHA by weight (about 400-500 mg DHA/g oil). In certain embodiments, the undiluted DHA oil can be enriched by cold fractionation (resulting in oil containing about 60% w/w of DHA triglyceride), which DHA fraction optionally can be transesterified, and subjected to further downstream processing to produce the active DHA of the invention. In some embodiments of the invention, downstream processing of the oil comprises distillation and/or silica refinement.

[0040] Thus, to produce oil from which DHA can be derived, in certain aspects, the following steps can be used: fermentation of a DHA producing microorganism; harvesting the biomass; spray drying the biomass; extracting oil from the biomass; refining the oil; bleaching the oil; chill filtering the oil; deodorizing the oil; and adding an antioxidant to the oil. In some embodiments, the microorganism culture can be progressively transferred from smaller scale fermenters to a production size fermenter. In some embodiments, following a controlled growth over a pre-established period, the culture can be harvested by centrifugation then pasteurized and spray dried. In certain embodiments, the dried biomass can be flushed with nitrogen and packaged before being stored frozen at -20 °C. In certain embodiments, the DHA oil can be extracted from the dried biomass by mixing the biomass with n-hexane or isohexane in a batch process which disrupts the cells and allows the oil and cellular debris to be separated. In certain embodiments, the solvent can then be removed.

[0041] In some embodiments, the crude DHA oil can then undergo a refining process to remove free fatty acids and phospholipids. The refined DHA oil can be transferred to a vacuum bleaching vessel to assist in removing any remaining polar compounds and pro-oxidant metals, and to break down lipid oxidation products. The refined and bleached DHA oil can undergo a final clarification step by chilling and filtering the oil to facilitate the removal of any remaining insoluble fats, waxes, and solids.

[0042] Optionally, the DHA can be deodorized under vacuum in a packed column, counter current steam stripping deodorizer. Antioxidants such as ascorbyl palmitate, alpha-tocopherol, and tocotrienols can optionally be added to the deodorized oil to help stabilize the oil. In some embodiments, the final, undiluted DHA oil is maintained frozen at -20°C until further processing.

[0043] In some embodiments, the DHA oil can be converted to DHA ester by methods known in the art. In some embodiments, DHA esters of the invention can be produced

from DHA oil by the following steps: cold fractionation and filtration of the DHA oil (to yield for example about 60% triglyceride oil); direct transesterification (to yield about 60% DHA ethyl ester); molecular distillation (to yield about 88% DHA ethyl ester); silica refinement (to yield about 90% DHA ethyl ester); and addition of an antioxidant.

[0044] In some embodiments, the cold fractionation step can be carried out as follows: undiluted DHA oil (triglyceride) at about 500 mg/g DHA is mixed with acetone and cooled at a controlled rate in a tank with -80°C chilling capabilities. Saturated triglycerides crystallize out of solution, while polyunsaturated triglycerides at about 600 mg/g DHA remain in the liquid state. The solids containing about 300 mg/g can be filtered out with a 20 micron stainless steel screen from the liquid stream containing about 600 mg/g DHA. The solids stream can then be heated (melted) and collected. The 600 mg/g DHA liquid stream can be desolventized with heat and vacuum and then transferred to the transesterification reactor.

[0045] In some embodiments, the transesterification step is carried out on the 600 mg/g DHA oil, wherein the transesterification is done via direct transesterification using ethanol and sodium ethoxide. The transesterified material (DHA-ethyl ester) can then be subject to molecular distillation and thus, further distilled (3 passes, heavies, lights, heavies) to remove most of the other saturated fatty acids and some sterols and non-saponifiable material. The DHA-ethyl ester (DHA-EE) can be further refined by passing it through a silica column.

[0046] In some embodiments of the method, the DHA composition used has a level of DHA that is at least 40 wt% of total wt of fatty acid content. In some embodiments, the weight % of the DHA in the composition of DHA is at least 50 wt% of total wt of fatty acid content, at least 60 wt% of total wt of fatty acid content; at least 70 wt% of total wt of fatty acid content; at least 80 wt% of total wt of fatty acid content; at least 85 wt% of total wt of fatty acid content; at least 90 wt% of total wt of fatty acid content; at least 95 wt% of total wt of fatty acid content; at least 96 wt% of total wt of fatty acid content; at least 97 wt% of total wt of fatty acid content; at least 98 wt% of total wt of fatty acid content; or at least 99 wt% of total wt of fatty acid content.

[0047] In some embodiments, DHA is present in an amount of about 35% to about 99.9% (w/w) of the total fatty acid content of the oil, about 40% to about 99% (w/w) of the total fatty acid content of the oil, about 45% to about 98% (w/w) of the total fatty acid content

of the oil, about 65% to about 99.9% (w/w) of the total fatty acid content of the oil, or about 85% to about 95% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA is present in an amount greater than about 65% (w/w) of the total fatty acid content of the oil, greater than about 85% (w/w) of the total fatty acid content of the oil, greater than about 90% (w/w) of the total fatty acid content of the oil, or greater than about 95% (w/w) of the total fatty acid content of the oil. In some embodiments, the oil can be diluted with other oils, such as sunflower oil, to achieve the desired concentration of fatty acids.

[0048] In some embodiments, the DHA is about 30% (w/w) or more of the total fatty acid content of the oil, about 30% to about 99.9% (w/w) of the total fatty acid content of the oil, about 35% to about 99.9% (w/w) of the total fatty acid content of the oil, about 35% to about 60% (w/w) of the total fatty acid content of the oil, about 35% to about 50% (w/w) of the total fatty acid content of the oil, about 37% to about 45% (w/w) of the total fatty acid content of the oil, or about 38% to about 43% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA is greater than about 35%, about 37%, about 38%, about 39% or about 40% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA is about 30% to about 99.5% (w/w) of the total fatty acid content of the oil, or about 40% to about 65% (w/w) of the total fatty acid content of the oil.

[0049] In some of these embodiments, the DHA comprises about 40% to about 45% (w/w) of the total fatty acid content of the oil. In some of these embodiments, the DHA comprises about 35% to about 45% (w/w) of the total fatty acid content of the oil. In some of embodiments, the DHA comprises about 55% to about 67% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA comprises greater than about 70% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA comprises about 85% to about 99.5% (w/w) of the total fatty acid content of the oil.

[0050] In some embodiments, the DHA is greater than about 80% (w/w) of the total fatty acid content of the oil, about 80% to 99.9% (w/w) of the total fatty acid content of the oil, about 85% to about 99% (w/w) of the total fatty acid content of the oil, about 87% to about 98% (w/w) of the total fatty acid content of the oil, or about 90% to about 97% (w/w) of the total fatty acid content of the oil. In some embodiments, the DHA is great

than about 95%, about 97%, about 98%, about 99% or about 99.5% (w/w) of the total fatty acid content of the oil.

[0051] In some embodiments, the DHA comprises about 35% to about 96% of the weight of the oil. In some embodiments, the DHA comprises about 38% to about 42% of the weight of the oil. In some embodiments, the DHA in the oil comprises about 35% to about 45% of the total weight of the oil. In some embodiments, the DHA in the oil comprises about 55% of the total weight of the oil. In some embodiments, the DHA in the oil comprises about 85% to about 96% of the total weight of the oil.

[0052] In some embodiments, the DHA is about 30% (w/w) or more of the total oil content of the oil, about 30% to about 99.9% (w/w) of the total oil content of the oil, about 35% to about 99.9% (w/w) of the total oil content of the oil, about 35% to about 60% (w/w) of the total oil content of the oil, about 35% to about 50% (w/w) of the total oil content of the oil, about 37% to about 45% (w/w) of the total oil content of the oil, or about 38% to about 43% (w/w) of the total oil content of the oil. In some embodiments, the DHA is greater than about 35%, about 37%, about 38%, about 39% or about 40% (w/w) of the total oil content of the oil. In some embodiments, the DHA is about 30% to about 99.5% (w/w) of the total oil content of the oil, or about 40% to about 65% (w/w) of the total oil content of the oil.

[0053] In some of these embodiments, the DHA comprises about 40% to about 45% (w/w) of the total oil content of the oil. In some of these embodiments, the DHA comprises about 35% to about 45% (w/w) of the total oil content of the oil. In some of embodiments, the DHA comprises about 55% to about 67% (w/w) of the total oil content of the oil. In some embodiments, the DHA comprises greater than about 70% (w/w) of the total oil content of the oil. In some embodiments, the DHA comprises about 85% to about 99.5% (w/w) of the total oil content of the oil.

[0054] In some embodiments, the DHA is greater than about 80% (w/w) of the total oil content of the oil, about 80% to 99.9% (w/w) of the total oil content of the oil, about 85% to about 99% (w/w) of the total oil content of the oil, about 87% to about 98% (w/w) of the total oil content of the oil, or about 90% to about 97% (w/w) of the total oil content of the oil. In some embodiments, the DHA is greater than about 95%, about 97%, about 98%, about 99% or about 99.5% (w/w) of the total oil content of the oil. With respect to comparison of DHA to total fatty acid content or total oil content, weight % can be

determined by calculating the area under the curve (AUC) using standard means, e.g., dividing the DHA AUC by the total fatty acid AUC.

[0055] As used herein, "or less" or "less than about" refers to percentages that include 0%, or amounts not detectable by current means. As used herein, "max" refers to percentages that include 0%, or amounts not detectable by current means.

The term "DHA" refers to docosahexaenoic acid, also known by its chemical name (all-Z)-4,7,10,13,16,19-docosahexaenoic acid, as well as any salts or derivatives thereof. Thus, the term "DHA" encompasses the free acid DHA as well as DHA alkyl esters and triglycerides containing DHA.

[0056] The term "EPA" refers to eicosapentaenoic acid, known by its chemical name (all Z) 5,8,11,14,17-eicosapentaenoic acid, as well as any salts or derivatives thereof. Thus, the term "EPA" encompasses the free acid EPA as well as EPA alkyl esters and triglycerides containing EPA. EPA is an D-3 polyunsaturated fatty acid. Typical content of omega-3 fatty acids found in fatty fish have a ratio of DHA to EPA ratio of 4:1 or less, w/w.

[0057] In some embodiments of the method, the oil or composition comprising DHA has a DHA to EPA ratio of 4:1 or less. In some embodiments, the oil or composition comprising DHA is substantially free of EPA. As used herein, an oil or composition comprising DHA that is "substantially free of EPA" refers to a preparation of an oil or composition comprising DHA in which EPA is less than 3% (w/w) of the total fatty acid content of the composition, less than 2% (w/w) of the total fatty acid content of the composition, less than 1% (w/w) of the total fatty acid content of the composition, less than 0.5% (w/w) of the total fatty acid content of the composition, less than 0.2% (w/w) of the total fatty acid content of the composition, or less than 0.01% (w/w) of the total fatty acid content of the composition. In some embodiments, the EPA is not detectable in the composition using techniques known in the art. In some embodiments, the oil or composition comprising DHA has no EPA. Compositions of DHA prepared as provided herein can also be administered as a therapeutic substantially free of EPA.

[0058] In some embodiments, the oil or composition comprising DHA is substantially free of arachidonic acid (ARA). The term "ARA" refers to the compound (all-Z)-5,8,11,14-eicosatetraenoic acid (also referred to as (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid), as well as any salts or derivatives thereof. Thus, the term "ARA"

encompasses the free acid ARA as well as ARA alkyl esters and triglycerides containing ARA. ARA is a D-6 polyunsaturated fatty acid. DHA is "substantially free of ARA" when ARA is less than about 3% (w/w) of the total fatty acid content of the composition. In some embodiments, ARA comprises less than about 2% (w/w) of the total fatty acid content of the composition, less than 1% (w/w) of the total fatty acid content of the composition, less than 0.5% (w/w) of the total fatty acid content of the composition, less than 0.2% (w/w) of the total fatty acid content of the composition, or less than 0.01% (w/w) of the total fatty acid content of the composition. In some embodiments, the composition has no detectable amount of ARA. Compositions of DHA prepared as provided herein can also be administered as a therapeutic substantially free of arachidonic acid (ARA).

[0059] In some embodiments, the oil or composition comprising DHA is substantially free of docosapentaenoic acid 22:5 n-6 (DPAn6). The term "DPAn6" refers to docosapentaenoic acid, omega 6, known by its chemical name (all-Z)-4,7,10,13,16-docosapentaenoic acid, as well as any salts or esters thereof. The term "DPAn6" encompasses the free acid DPAn6 as well as DPAn6 alkyl esters and triglycerides containing DPAn6. DPAn6 is a D-6 polyunsaturated fatty acid. DHA is "substantially free of DPAn6" when DPAn6 is less than about 3% (w/w) of the total fatty acid content of the composition. In some embodiments, DPAn6 comprises less than about 2% (w/w) of the total fatty acid content of the composition, less than 1% (w/w) of the total fatty acid content of the composition, less than 0.5% (w/w) of the total fatty acid content of the composition, less than 0.2% (w/w) of the total fatty acid content of the composition, or less than 0.01% (w/w) of the total fatty acid content of the composition. In some embodiments, the composition has no detectable amount of DPAn6. Compositions of DHA prepared as provided herein can also be administered as a therapeutic substantially free of DPAn6.

[0060] In some embodiments, the composition of the present invention does not contain a measurable amount of docosapentaenoic acid 22:5n-3 (DPAn3); docosapentaenoic acid 22:5n-6 (DPAn6); and/or 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8).

[0061] In some embodiments, the composition of DHA may include an additional lipid. As used herein, the term "lipid" includes phospholipids (PL); free fatty acids; esters of fatty acids; triacylglycerols (TAG); diacylglycerides; monoacylglycerides; phosphatides;

waxes (esters of alcohols and fatty acids); sterols and sterol esters; carotenoids; xanthophylls (e.g., oxycarotenoids); hydrocarbons; and other lipids known to one of ordinary skill in the art. The lipid can be chosen to have minimal adverse health effects or minimally affect the effectiveness of DHA when administered in combination with DHA.

[0062] In some embodiments, the composition of DHA may include an additional unsaturated lipid. In some embodiments, the unsaturated lipid is a polyunsaturated lipid, such as an omega-3 fatty acid or omega-6 fatty acid. An exemplary omega-6 fatty acid that may be used in the composition is docosapentaenoic acid (DPA), including DPA (n-6) or DPA (n-3).

[0063] In the methods and compositions herein, additional fatty acids can be present in the oil or composition. These fatty acids can include fatty acids that are not removed during the purification process, i.e., fatty acids that are co-isolated with DHA from an organism. In some embodiments, one or more non-DHA fatty acids can be added to the oil to achieve a desired concentration of specific non-DHA fatty acids. Any of these fatty acids can be present in various concentrations. For example, in some embodiments, the oil comprises 0.01% to about 4% (w/w) of oleic acid. In some embodiments, the oil comprises 0.01% to 0.5% (w/w) of one or more of the following fatty acids: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) heptadecanoic acid; (g) stearic acid; (h) oleic acid; (i) linoleic acid; (j) α-linolenic acid; (k) arachidic acid; (l) eicosenoic acid; (m) arachidonic acid; (n) erucic acid; (o) docosapentaenoic acid 22:5n-3 (DPAn3); and (p) nervonic acid. In some embodiments, the oil or composition comprises 0.01% to 0.1% (w/w) of one or more of the following fatty acids: (a) lauric acid; (b) heptadecanoic acid; (c) stearic acid; (d) arachidic acid; (e) eicosenoic acid; and (f) arachidonic acid. In some embodiments, the oil or composition comprises less than 0.5% (w/w) each of the following fatty acids: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) heptadecanoic acid; (g) stearic acid; (h) linoleic acid; (i) α-linolenic acid; (j) arachidic acid; (k) eicosenoic acid; (l) arachidonic acid; (m) erucic acid; (n) docosapentaenoic acid 22:5n-3 (DPAn3); and (o) nervonic acid. In some embodiments, the oils of the present invention do not contain a measurable amount of one or more of the following fatty acids: (a) capric

acid; (b) linoleic acid; (c) α-linolenic acid; and (d) docosapentaenoic acid 22:5n-3 (DPAn3).

[0064] In some embodiments, the oil comprises 0.1% to 60% (w/w) of one or more of the following fatty acids, or esters thereof: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid, (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) α-linolenic acid; (j) docosapentaenoic acid 22:5n-3 (DPAn3); (k) docosapentaenoic acid 22:5n-6 (DPAn6); and (l) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8). In some embodiments, the oil comprises 20% to 40% (w/w) of one or more of the following fatty acids, or esters thereof: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) a-linolenic acid; j) docosapentaenoic acid 22:5n-3 (DPAn3); (k) docosapentaenoic acid 22:5n-6 (DPAn6); and (1) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8). In some embodiments, the oil comprises less than 1% (w/w) each of the following fatty acids, or esters thereof: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid, (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) α-linolenic acid; (j) docosapentaenoic acid 22:5n-3 (DPAn3); (k) docosapentaenoic acid 22:5n-6 (DPAn6); and (l) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8).

[0065] In some embodiments the oil comprises 0.1% to 20% of one or more of the following fatty acids: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) a-linolenic acid; (j) DPA n-3 (22:5, n-3); (k) DPA n-6 (22:5, n-6); and (1) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8). In some embodiments, the oil comprises 1% to 5% of one or more of the following fatty acids: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) a-linolenic acid; (j) DPA n-3 (22:5, n-3); (k) DPA n-6 (22:5, n-6); and (1) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8). In some embodiments, the dosage form comprises less than 1% each of the following fatty acids: (a) capric acid; (b) lauric acid; (c) myristic acid; (d) palmitic acid; (e) palmitoleic acid; (f) stearic acid; (g) oleic acid; (h) linoleic acid; (i) a-linolenic acid; (j) docosapentaenoic acid 22:5n-3, 22:5w3 (DPAn3); (k) docosapentaenoic acid 22:5n-6, 22:5w6 (DPAn6); and (1) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8).

[0066] In some of embodiments of DHA composition described herein, the dosage form is characterized by one or more the following fatty acids (or esters thereof). The embodiments provided herein may further comprise about 2% or less (w/w) of capric acid (C10:0). The embodiments herein may further comprise about 6% or less (w/w) of lauric acid (C12:0). The embodiments herein may further comprise about 20% or less (w/w), or about 5% to about 20% (w/w) of myristic acid (C14:0). The embodiments herein may further comprise about 20% (w/w) or less, or about 5% to about 20% (w/w) of palmitic acid (C16:0). The embodiments herein may further comprise about 3% (w/w) or less of palmitoleic acid (C16:1n-7). The embodiments herein may further comprise about 2% (w/w) or less of stearic acid (C18:0). The embodiments herein may further comprise about 40% (w/w) or less, or about 10% to about 40% (w/w) of oleic acid (C18:1n-9). The embodiments herein may further comprise about 5% (w/w) or less of linoleic acid (C18:2). The embodiments herein may further comprise about 2% (w/w) or less of nervonic acid (C24:1). The embodiments herein may further comprise about 3% (w/w) or less of other fatty acids or esters thereof. The DHA dosage form with the preceding characteristics may comprise DHASCO®, an oil derived from *Crypthecodinium cohnii* containing docosahexaenoic acid (DHA).

[0067] An exemplary DHA (triglyceride) containing oil derived from *Crypthecodinium cohnii* is characterized by the specified amount of components listed in **Table 1,** where "Max" refers to the amount of the component that can be present up to the specified amount.

Table 1

Fatty Acids	Concentration (w/w)
10:0	Max 2%
12:0	Max 6%
14:0	5-20%
16:0	5-20%
16:1	Max 3%
18:0	Max 2%
18:1	10-40%
18:2	Max 5%
22:6 DHA	40-45%
24:1	Max 2%
Others	Max 3%
Elemental Composition	

Fatty Acids	Concentration (w/w)
Arsenic	Max 0.5 ppm
Copper	Max 0.1 ppm
Iron	Max 0.5 ppm
Lead	Max 0.2 ppm
Mercury	Max 0.04 ppm
Phosphorous	Max 10 ppm
Chemical Characteristics	
Peroxide value	Max 5 meq/kg
Free fatty acid	Max 0.4%
Unsaponifiable Matter	Max 3.5%

[0068] An exemplary undiluted DHA (triglyceride) containing oil derived from *Crypthecodinium cohnii* is characterized by amount of DHA described herein, and one or more, or all of the features listed below in **Table 2**, where "Max" refers to the amount of the component that can be present up to the specified amount.

Table 2: Characteristics of Undiluted DHA Oil

Component	**Concentration (w/w)**
DHA content mg/DHA/g oil	Min 480 mg/g
Free Fatty Acid	Max.0.4%
Peroxide Value (PV)	Max. 5 meq/kg
Anisidine Value (AV)	Max 20
Moisture and Volatiles (M & V)	Max. 0.02%
Unsaponifiable Matter	Max. 3.5%
Insoluble Impurities	Max. 0.1%
Trans Fatty Acid	Max. 1%
Arsenic	Max. 0.5 ppm
Cadmium	Max. 0.2 ppm
Chromium	Max. 0.2 ppm
Copper	Max. 0.1 ppm
Iron	Max. 0.5 ppm
Lead	Max. 0.2 ppm
Manganese	Max. 0.04 ppm
Mercury	Max. 0.04 ppm
Molybdenum	Max. 0.2 ppm
Nickel	Max.0.2 ppm
Phosphorus	Max. 10 ppm
Silicon	Max. 500 ppm
Sulfur	Max. 100 ppm
18:1 n-9 Oleic Acid	Max. 10%
20:5 n-3 EPA	Max. 0.1%
Unknown Fatty Acids	Max. 3.0%

[0069] In some embodiments, an oil is characterized by one or more the following fatty acids (or esters thereof), expressed as wt% of the total fatty acid content. The embodiments provided herein may further comprise about 2% or less (w/w) of capric acid (C10:0). The embodiments provided herein may further comprise about 6% or less (w/w) of lauric acid (C12:0). The embodiments provided herein may further comprise about 20% or less, or about 10 to about 20% (w/w) of myristic acid (C14:0). The embodiments provided herein may further comprise about 15% or less, or about 5 to about 15% (w/w) of palmitic acid (C16:0). The embodiments provided herein may further comprise about 5% or less (w/w) of palmitoleic acid (C16:1n-7). The embodiments provided herein may further comprise about 2% or less (w/w) of stearic acid (C18:0). The embodiments provided herein may further comprise about 20% or less, or about 5% to about 20% (w/w) of oleic acid (C18:1n-9). The embodiments provided herein may further comprise about 2% or less (w/w) of linoleic acid (C18:2). The embodiments provided herein may further comprise about 2% or less (w/w) of nervonic acid (C24:1). The embodiments provided herein may further comprise about 3% or less (w/w) of other fatty acids. An oil with the preceding characteristics may be an oil derived from *Crypthecodinium cohnii* containing docosahexaenoic acid (DHA).

[0070] In some embodiments, the oil comprises, measured in percentage of free fatty acid, about 35-65%, 40-55%, 35-57%, or 57-65% (w/w) DHA (22:6 n-3); about 0-2% (w/w) capric acid (10:0); about 0-6% (w/w) lauric acid (12:0); about 10-20% (w/w) myristic acid (14:0); about 5-15% (w/w) palmitic acid (16:0); about 0-5% (w/w) palmitoleic acid (16:1); about 0-2% (w/w) stearic acid (18:0); about 5-20% or 5-25% (w/w) oleic acid (18:1); about 0-2% (w/w) linoleic acid (18:2); and about 0-2% (w/w) nervonic acid (24:1, n-9). In one embodiment, such an oil is from a microorganism of the genus *Thraustochytrium*. In another embodiment, the free fatty acid content is less than 0.4%.

[0071] The present invention also provides compositions comprising at least about 40 wt% DHA and at least about 0.1 wt % of DPA (n-3). In some embodiments, the compositions comprise at least about 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, or 65 wt% DHA, optionally in triglyceride form, as a percentage of total fatty acids.

[0072] An exemplary DHA containing oil derived from *Crypthecodinium cohnii* is characterized by the specified amount of components listed in **Table 3,** where "Max" refers to the amount of the component that can be present up to the specified amount.

Table 3

Fatty Acid	**Concentration (w/w)**
10:0	0-2%
12:0	0-6%
14:0	10-20%
16:0	5-15%
16:1	0-5%
18:0	0-2%
18:1	5-20%
18:2	0-2%%
22:6 (n-3) DHA	57-65%
24:1	0-2%
Others	0-3%
Elemental Composition	
Arsenic	Max 0.5 ppm
Copper	Max 0.1 ppm
Iron	Max 0.5 ppm
Lead	Max 0.2 ppm
Mercury	Max 0.2 ppm
Phosphorous	Max 10 ppm
Chemical Characteristics	
Peroxide value	Max 5 meq/kg
Free fatty acid	Max 0.4%
Unsaponifiable Matter	Max 3.5%
Trans fatty acids	<3.5%
Moisture and Volatiles	<0.1%
Insoluble impurities	<0.1%

[0073] In some embodiments, an oil is characterized by one or more the following fatty acids (or esters thereof), expressed as wt% of the total fatty acid content. The embodiments provided herein may further comprise about 0.1% or less (w/w) of myristic acid (C14:0) or is not detectable. The embodiments provided herein may further comprise about 0.5% or less (w/w) of palmitic acid (C16:0). The embodiments provided herein may further comprise about 0.5% or less (w/w) of palmitoleic acid (C16:1n-7). The embodiments provided herein may further comprise about 0.5% or less (w/w) of stearic acid (C18:0), or is not detectable. The embodiments provided herein may further comprise about 4% or less (w/w) of oleic acid (C18:1n-9). The embodiments provided

herein may further comprise less than 0.1% (w/w) of linoleic acid (C18:2) or is not detectable. The embodiments provided herein may further comprise less than 0.1% (w/w) of eicosapentaenoic acid (C20:5) or is not detectable. The embodiments provided herein may further comprise about 2% or less (w/w) of decosapentaenoic acid (22:5n-3). The embodiments provided herein may further comprise about 1% or less (w/w) of octacosaoctaenoic acid (28:8 n-3). The embodiments provided herein may further comprise about 0.5% or less (w/w) of tetracosaenoic acid (24:1n9). The embodiments provided herein may further comprise about 1% or less (w/w) of other fatty acids. The DHA in the oil with the preceding characteristics may be in the form of a DHA ester, preferably an alkyl ester, such as a methyl ester, ethyl ester, propyl ester, or combinations thereof, prepared from an algal oil prepared from the Crypthecodinium, *cohnii sp.*

[0074] In some embodiments, the DHA composition may comprise DHASCO®. DHASCO® is an oil derived from *Crypthecodinium cohnii* containing high amounts of docosahexaenoic acid (DHA), and more specifically contains the following approximate exemplary amounts of these fatty acids, as a percentage of the total fatty acids: myristic acid (14:0) 10-20%; palmitic acid (16:0) 10-20%; palmitoleic acid (16:1) 0-2%; stearic acid (18:0) 0-2%; oleic acid (18:1) 10-30%; linoleic acid (18:2) 0-5%; arachidic acid (20:0) 0-1%; behenic acid (22:0) 0-1%; docosapentaenoic acid (22:5) 0-1%; docosahexanoic acid (22:6) (DHA) 40-45%; nervonic acid (24:1) 0-2%; and others 0-3%.

[0075] The present invention also provides compositions comprising at least about 40 wt% DHA and at least about 0.1 wt% of 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8). In some embodiments, the compositions comprise at least about 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, or 65 wt% DHA, optionally in triglyceride form, as a percentage of total fatty acids. In other embodiments, the compositions comprise at least about 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt% of DHA, optionally in ethyl ester form, as a percentage of total fatty acids. In certain embodiments, the amount of C28:8 in the compositions may be at least about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 or 1.5 wt%. The C28:8 may be present in any form, including triglyceride or ester form. For example, the C28:8 may be present in ethyl ester form.

[0076] In some embodiments, an oil is characterized by one or more the following fatty acids (or esters thereof), expressed as wt% of the total fatty acid content. The embodiments provided herein may further comprise about 12% or less, or about 6% to

about 12% (w/w) of myristic acid (C14:0). The embodiments provided herein may further comprise about 28% or less, or about 18 to about 28% (w/w) of palmitic acid (C16:0). The embodiments provided herein may further comprise about 2% or less (w/w) of stearic acid (C18:0). The embodiments provided herein may further comprise about 8% or less of (w/w) oleic acid (C18:1n-9). The embodiments provided herein may further comprise about 2% or less (w/w) of linoleic acid (C18:2). The embodiments provided herein may further comprise about 2% or less (w/w) of arachidonic acid (C20:4). The embodiments provided herein may further comprise about 3% or less (w/w) of eicosapentaenoic acid (C20:5). The embodiments provided herein may further comprise about 18% or less, or about 12% to about 18% (w/w) of decosapentaenoic acid (22:5n-6). The embodiments provided herein may further comprise about 10% or less (w/w) of other fatty acids. In some of these embodiments, the ratio of wt% of DHA to wt% of DPAn6 is about 2.5 to about 2.7. An oil with the preceding characteristics may comprise Life's DHA™ (also formerly referenced as DHA™-S and DHASCO®-S), Martek Biosciences, Columbia, MD), an oil derived from the Thraustochytrid, *Schizochytrium sp.*, that contains a high amount of DHA and also contains docosapentaenoic acid (n-6) (DPAn-6).

[0077] In some embodiments, more specifically, DHA™-S contains the following approximate exemplary amounts of these fatty acids, as a percentage of total fatty acids: myristic acid (14:0) 8.71%; palmitic acid (16:0) 22.15%; stearic acid (18:0) 0.66%; linoleic acid (18:2) 0.46%; arachidonic acid (20:4) 0.52%; eicosapentenoic acid (20:5, n-3) 1.36%; docosapentaenoic acid (22:5, n-6) (DPAn-6) 16.28%; docosahexaenoic acid (DHA) (22:6, n-3) 41.14%; and others 8%.

[0078] In some embodiments, the oil comprises, measured in percentage of free fatty acid, about 35-45% DHA (22:6 n-3); about 0-2% lauric acid (12:0); about 5-10% myristic acid (14:0); about 5-20% palmitic acid (16:0); about 0-5% palmitoleic acid (16:1); about 0-5% stearic acid (18:0); about 0-5% vaccenic acid or oleic acid (18:1 n-7 and n-9, respectively); about 0-2% linoleic acid (18:2, n-6); about 0-5% stearidonic acid (18:4 n-3); about 0-10% arachidonic acid (20:4 n-3, n-5, or n-6); about 0-2% adrenic acid (22:4 n-6); about 0-5% DPA n-3 (22:5); about 10-25% DPA n-6 (22:5); and 0-2% 24:0. In one embodiment, such an oil is from a microorganism of the genus *Schizochytrium*.

[0079] An exemplary DHA (triglyceride) containing oil derived from *Schizochytrium* sp. is characterized by the specified amount of components listed in Table 5, where "Max" refers to the amount of the component that can be present up to the specified amount.

Table 4

Fatty Acids	**Concentration (w/w)**
14:0	6.0-12.0%
16:0	18-28%
18:0	Max 2%
18:1	Max 8%
18:2	Max 2%
20:4 ARA	Max 2%
20:5 (n-3) EPA	Max 3%
22:5 (n-6) DPA	12-18%
22:6 (n-3) DHA	Min 35%
Others	Max 10%
Elemental Composition	
Arsenic	Max 0.2 ppm
Copper	Max 0.05 ppm
Iron	Max 0.2 ppm
Lead	Max 0.1 ppm
Mercury	Max 0.04 ppm
Chemical Characteristics	
Peroxide value	Max 5 meq/kg
Free fatty acid	Max 0.25%
Moisture and Volatiles	Max 0.05%
Unsaponifiable Matter	Max 4.5%
Trans fatty acids	Max 1%

[0080] Compositions useful in the methods herein also include compositions that comprise at least about 90 wt% of a combination of DPA (n-6) and DHA. In certain embodiments, the compositions may comprise at least about 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt% of a combination of DPA (n-6) and DHA. In some embodiments, the compositions may comprise at least about 10 wt% DHA and at least about 10 wt% DPA (n-6). In other embodiments, the compositions may comprise at least about 15 or 20 wt% DHA and at least about 15 or 20 wt% DPA (n-6).

[0081] The present invention also provides compositions comprising at least about 90 wt% of a combination of DPA (n-6) and DHA, and at least one additional fatty acid or a derivative, such as an ester, thereof. In certain embodiments, the compositions may comprise at least about 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt% of a combination of DPA

(n-6) and DHA. In some embodiments, the additional fatty acid may have a boiling point of about 150-170°C at a pressure of 0.8 mm Hg.

[0082] The DHA/DPA (n-6) compositions described above may further comprise less than about 4% of a saturated fatty acid or an ester thereof. In certain embodiments, the compositions may comprise less than about 3.5%, 3.0%, 2.5%, 2.0%, 1.5%, 1.0% or 0.5% of a saturated fatty acid or a derivative thereof.

[0083] In some embodiments, the composition or oil is characterized by one or more of the following fatty acids (or esters thereof, particularly ethyl esters), expressed as wt% of the total fatty acid content. The embodiments provided herein may further comprise about 0.5% or less (w/w) of lauric acid (C12:0). The embodiments provided herein may further comprise about 2% or less (w/w) of myristic acid (C14:0). The embodiments provided herein may further comprise about 0.5% or less (w/w) of myristoleic acid (C14:1). The embodiments provided herein may further comprise about 1% or less of palmitic acid (C16:0). The embodiments provided herein may further comprise about 1% or less (w/w) of linoleic acid (C18:2) (n-6). The embodiments provided herein may further comprise about 3% or less (w/w) of dihomo gamma linolenic acid (C20:3) (n-6). The embodiments provided herein may further comprise about 0.5% or less (w/w) of eicosatrienoic (C20:3) (n-3). The embodiments provided herein may further comprise about 1% or less (w/w) of arachidonic acid (C20:4). The embodiments provided herein may further comprise about 3% or less (w/w) of eicosapentaenoic acid (C20:5) (n-3). The embodiments provided herein may further comprise about 3% or less (w/w) of docosatrienoic acid (22:3). The embodiments provided herein may further comprise about 27% or less (w/w) of decosapentaenoic acid (22:5) (n-6). The embodiments provided herein may further comprise about 10% or less (w/w) of other components. In some of these embodiments, the ratio of wt% of DHA to wt% of DPAn6 is about 2.5 to about 2.7. An oil with the preceding characteristics may comprise ethyl ester oil derived from the oil of Thraustochytrid, *Schizochytrium sp.*

[0084] In some embodiments, the present invention further includes use of compositions comprising at least about 70 wt% DHA and at least about 15, 20, or 25 wt% DPA (n-6).

[0085] In some embodiments, the saturated fatty acid or an ester thereof may contain less than 20 carbons, such as, for example, a saturated fatty acid or an ester thereof that

contains 19, 18, 17. 16, 15, 14, 13, 12, 11, 10, 9 or 8 carbons. In certain embodiments, the saturated fatty acid or ester thereof may contain 14 or 16 carbons.

[0086] Compositions prepared by the methods provided herein may comprise greater than 90% DHA free fatty acid and ethyl esters.

EXAMPLES

1.1 Preparation of DHA Free Fatty Acid

1.1.1 Preparation of Crude DHA Free Fatty Acid

[0087] 1.1.1.1 Method 1 (with solvent extraction) To a multi-neck round-bottom flask equipped with proper stirring mechanism and nitrogen protection is added a crude DHA-containing oil (substantially as described in Table 3) followed by a solution of sodium hydroxide (0.1864 g/g oil) in water (0.5-1 ml/g oil) and ethanol (1-2 ml/g oil). The resulting reaction mixture is heated at 50 °C for 3 hours and then acetone (5-20 ml/g oil) is slowly added. (Caution: fire hazard.) The solid-liquid mixture is stirred at 2-16 hours at ambient temperature to -20 °C. The precipitate is filtered off and rinsed with acetone (10% of acetone used earlier). The filtrate is evaporated *in vacuo* at 25 °C to 1/6 of the original volume. The concentrated aqueous mixture is diluted with water (1.5 ml/g oil) and acidified with 10 N sulfuric acid (*ca* 0.25 ml/g oil). The resulting mixture is stirred at room temperature for 2 hours and extracted with hexanes (0.75 ml/g oil, twice). The combined hexanes extracts are washed with saturated sodium chloride (0.25 ml/g oil, twice) and then filtered. The filtrate is evaporated *in vacuo* at 40 °C and chased with hexanes (0.5 ml/g oil). The oil-like liquid is pumped under high vacuum overnight at room temperature to give a crude DHA free fatty acid with a FAME area percentage of 92-99% in a DHA-based yield of 50-81%.

[0088] 1.1.1.2 Method 2 (without solvent extraction) To a multi-neck round-bottom flask equipped with proper stirring mechanism and nitrogen protection is added a crude DHA-containing oil (as described in Table 3) followed by a solution of sodium hydroxide (0.1864 g/g oil) in water (0.5-1 ml/g oil) and ethanol (1-2 ml/g oil). The resulting reaction mixture is heated at 50 °C for 3 hours and then acetone (5-20 ml/g oil) is slowly added. (Caution: fire hazard.) The solid-liquid mixture is stirred for 2-16 hours at ambient temperature to -20 °C. The precipitate is filtered off and rinsed with acetone (10% of

acetone used earlier). The filtrate is evaporated *in vacuo* at 25 °C to 1/6 of the original volume. The concentrated aqueous mixture is diluted with water (1.5 ml/g oil) and acidified with 10 N sulfuric acid *(ca* 0.25 ml/ g oil). The resulting mixture is stirred at room temperature for 2 hours. The top oil layer is split, washed with saturated sodium chloride (0.25 ml/g oil, twice), evaporated *in vacuo* at 40 °C, and pumped overnight under high vacuum at room temperature to give a crude DHA free fatty acid with a FAME area percentage of 92-99% in a DHA-based yield of 50-81%.

1.1.2 Purification of DHA Free Fatty Acid

[0089] 1.1.2.1 Method 1-Short Path Distillation The crude DHA free fatty acid prepared above is distilled at 150-210 °C with a vacuum below 1 torr and the DHA-containing fraction is collected to give purified DHA free fatty acid with a FAME area percentage of 92-99% in a DHA-based recovery of 60-90%.

[0090] 1.1.2.2 Method 2-HPLC/Column Chromatography The prepared crude DHA free fatty acid is chromatographed on silica gel with hexanes to 20% (v/v) ethyl acetate in hexanes containing 1% acetic acid as eluent to give high-potency DHA free fatty acid with FAME area percentages of 92-99% in a DHA-based recovery of 65-95%.

2.1 Preparation DHA Ethyl Ester

[0091] 2.2.1 Method 1 (from purified DHA FFA) To a solution of purified DHA FFA prepared above in anhydrous dichloromethane (8 ml/g DHA FFA) in a water bath is added 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.0 eq) followed by triethylamine (1.0 eq), 4-*N*,*N*-dimethylaminopyridine (DMAP, 0.02 eq), and absolute ethanol (2 ml/g DHA FFA). The reaction mixture is stirred overnight at ambient temperature and evaporated *in vacuo*. The crude residue is taken up with hexanes (10 ml/g DHA FFA) and washed with 0.1 N sulfuric acid (10 ml/g DHA FFA) and water (10 ml/g DHA FFA, twice). The hexanes solution is dried over sodium sulfate, mixed with silica gel (0.1 g/g DHA FFA) and decolorizing carbon (0.1 g/g DHA FFA) for 1 hour, and filtered. The filtrate is evaporated *in vacuo* at 40 °C. The residue is pumped 16 hours under high vacuum to give a DHA ethyl ester with an area percentage of 98% for a yield of 93%.

[0092] 2.2.2 Method 2 (from purified DHA FFA) To a solution of purified DHA FFA (prepared above) in anhydrous hexanes (8 ml/g DHA FFA) containing a catalytic amount of *N,N*-dimethylformamide (DMF, 2 drops per 50 mmol of DHA FFA) in an ice/water bath is added dropwise oxalyl chloride (1.05 eq). 10 min later the cooling bath is removed and the reaction mixture is stirred at room temperature for 2 hrs. The mixture is cooled back to 0 °C and absolute ethanol (7 eq) is slowly added. 10 min later triethylamine (1.2 eq) is added dropwise. The resulting mixture is stirred at room temperature for 16 hours and diluted with water (10 ml/g DHA FFA). The organic layer is split and washed with saturated sodium bicarbonate (10 ml/g DHA FFA) and brine (10 ml/g DHA FFA). The top hexanes layer is dried over sodium sulfate, mixed with silica gel (0.1 g/g DHA FFA) and decolorizing carbon (0.1 g/g DHA FFA) for 1 hour, and filtered. The filtrate is evaporated *in vacuo* at 40 °C. The residue is pumped 16 hours under high vacuum to give a DHA ethyl ester with an area percentage of 97% with a yield of 97%.

[0093] 2.2.3 Method 3 (from purified DHA FFA) To the solution of purified DHA FFA prepared above in absolute ethanol (5 ml/g DHA FFA) in an ice/water bath is added dropwise thionyl chloride (1.2 eq). 10 min later the cooling bath is removed and the reaction mixture is stirred for 16 hours at room temperature. The reaction mixture is cooled below 10 °C in an ice/water bath and saturated sodium bicarbonate (10 ml/g DHA FFA) is slowly added. The mixture is extracted with hexanes (10 ml/g DHA FFA, twice). The combined hexanes extracts are washed with saturated sodium bicarbonate (10 ml/g DHA FFA) and brine (10 ml/g DHA FFA), dried over sodium sulfate, mixed with silica gel (0.1 g/g DHA FFA) and decolorizing carbon (0.1 g/g DHA FFA) for 1 hour, and filtered. The filtrate is evaporated *in vacuo* at 40 °C. The oil is pumped 16 hours under high vacuum to give a DHA ethyl ester with an area percentage of 96% with a quantitative yield.

[0094] 2.2.4 Method 4 (from crude DHA FFA) The crude DHA FFA prepared in section 2.1.1 is converted to ethyl ester *via* method 1, 2, or 3 in section 2.2. The ethyl ester thus prepared is purified by short path distillation at 150-180 °C or by column chromatography as mentioned in section 2.1.2.2 to give purified DHA ethyl ester in a comparable yield and purity.

Table 5: Data summary of hydrolysis of DHA algal oil (triglycerides) experiments as prepared substantially according to Methods 1 and 2 of Section 1.1.1 (except Experiment 1) (in situ crystallization)

Experiment	Amount (g)	EtOH (mL/g)	Water (mL/g)	NaOH (g)	Acetone (L)	Cryst. T (°C)	Cryst. time (hr)	Recovery (%) (Oil Based)	Recovery (%) (DHA Based)	Potency (mg/g)	% DHA	% Fat
1	50	2	1	5	1	-20	16	38	95	857	91	92.7
2	100	2	1	10	2	-20	24	29	73	832	95.6	90.35
3	100	2	1	18.64	2	-20	16	24	49	812	99.14	85.25
4	200	2	1	37.28	4	-20	16	23	46	808	98.74	85.06
5	100	2	1	18.64	2	-20	16	25	44	721	98.58	76.01
6	100	2	1	18.64	2	-20	16	28	54	758	99.14	79.8
7	100	2	1	18.64	2	20	16	36	55	610	97.34	64.6
8	100	2	1	18.64	2	6	16	27	43	636	95.23	68.64
9	100	1	0.5	18.64	1	20	16	23	50	892	96.5	95.4
10	100	1.5	0.75	18.64	1.5	20	16	30	58	771	92.2	86.36
11	100	2	1	18.64	1.5	20	16	25	49	785	89.55	90.48
12	100	2	1	18.64	1	20	16	22	45	811	88.68	94.81
13	200	2	1	37.28	4	20	16	45	62	687	92.57	74.86
14	200	2	1	37.28	4	20	16	46	74	646	94.84	69.76
15	200	2	1	37.28	4	20	16	40	74	732	92.23	86.6
16	200	2	1	37.28	4	20	16	54	89	655	92.28	73.51
17	200	1	0.5	37.28	2	20	16	39	72	748	94.18	80.96
18	200	1.25	0.625	37.28	2.5	20	16	38	76	800	93.24	84.01
19	200	1.5	0.75	37.28	3	20	16	27	53	785	96.52	82.87
20	200	1.5	0.75	37.28	3	20	16	38	77	820	94.45	87.33
21	200	1.5	0.75	37.28	3	20	16	40	78	785	94.61	83.43
22	200	1.75	0.875	37.28	3.5	20	16	43	81	754	91.66	80.5
23	200	2	1	37.28	4	20	16	38	77	797	94.86	85.71
24	200	1	0.5	37.28	1	20	16	33	66	803	94.08	86.57
25	200	1	0.5	37.28	2	20	16	36	64	715	97.32	74.14
26	200	1	0.5	37.28	3	20	16	36	64	715	97.92	73.66
27	200	1	0.5	37.28	4	20	16	32	59	742	98.24	76.62

Table 6: Fatty Acid Profile (Experiment 21)

Sample	A	B
mg/g DHA	782.8	786.0
% Fat (by mg/g DHA)	88.33	88.69
Fatty Acid Profile:		
% 08:0	0.10	0.10
% 09:0	0.00	0.00
% 10:0	0.38	0.38
% 11:0	0.00	0.00
% 11:1	0.00	0.00
% 12:0	1.04	1.04
% 12:1	0.00	0.00
% 13:0	0.00	0.00
% 13:1	0.00	0.00
% 14:0	1.21	1.21
% 14:1	0.00	0.00
% 15:1	0.00	0.00
% 16:0	0.50	0.51
% 16:1	1.59	1.60
% 16:2	0.00	0.00
% 16:3	0.00	0.00
% 17:0	0.00	0.00
% 18:0	0.00	0.00
% 18:1 n-9	2.80	2.82
% 18:1 n-7	0.00	0.00
% 18:2	0.00	0.00
% 18:3 n-6	0.00	0.00
% 18:3 n-3 ALA	0.00	0.00
% 18:4 n-3	0.00	0.00
% 20:0	0.00	0.00
% 20:1 n-9	0.00	0.00
% 20:2	0.00	0.00
% 20:3 n-9	0.00	0.00
% 20:3 n-6	0.00	0.00
% 20:3 n-3	0.00	0.00
% 20:4 n-3 ARA	**0.00**	**0.00**
% 20:5 n-3 EPA	**0.00**	**0.00**
% 22:0	0.00	0.00
% 22:1	0.00	0.00
% 22:2	0.00	0.00
% 22:3	0.00	0.00
% 22:4 n-6	0.00	0.00
% 22:5 n-6	0.00	0.00
% 22:5 n-3 DPA	**0.82**	**0.80**
% 22:6 n-3 DHA	**91.05**	**91.04**
% 24:0	0.00	0.00
% 24:1	0.00	0.00
% Unknown	0.50	0.50

WHAT IS CLAIMED IS:

1. Provided herein is a process for making a free fatty acid of a polyunsaturated fatty acid (PUFA) comprising:

(a) adding acetone to a PUFA salt solution to form a precipitate wherein the acetone is added before any significant acidifying step is performed;

(b) separating the precipitate from the solution; and

(c) acidifying the solution to produce a free fatty acid PUFA.

2. A process for making a free fatty acid of a polyunsaturated fatty acid (PUFA) comprising:

(a) saponifying a PUFA triglyceride to form a PUFA salt solution;

(b) adding acetone to the salt solution to form a precipitate wherein the acetone is added before any significant acidifying step;

(c) filtering the precipitate to form a filtrate; and

(d) acidifying the filtrate to produce a free fatty acid PUFA.

3. A composition comprising a PUFA selected from a free fatty acid or a ester wherein the PUFA is made according to a process comprising:

(a) adding acetone to a PUFA salt solution to form a precipitate wherein the acetone is added before any significant acidifying step is performed;

(b) separating the precipitate from the solution; and

(c) acidifying the solution to produce a free fatty acid PUFA.

4. A composition comprising a PUFA selected from a free fatty acid or a ester wherein the PUFA is made according to a process comprising:

(a) saponifying a PUFA triglyceride to form a PUFA salt solution;

(b) adding acetone to the salt solution to form a precipitate wherein the acetone is added before any significant acidifying step;

(c) filtering the precipitate to form a filtrate; and

(d) acidifying the filtrate to produce a free fatty acid PUFA.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/25666

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 37/00; A61K 31/20; C12P 7/64 (2012.01)
USPC - 435/134; 514/560
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC-435/134; 514/560

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC-424/451; 426/545-546, 601; 554/175 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PUBWEST, Google, Google Scholar
Search terms: Polyunsaturated fatty acids, PUFA, omega-3, omega-6, omega-9 fatty acids, acetone, saponify, saponification, hydrolysis, hydrolyze, acidify, crystallize, crystallization, fractionate, fractionization, separation, precipitate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,846,942 B2 (Rubin) 25 January 2005 (25.01.2005) entire document, especially col 3, ln 55-58; col 4, ln 1-8	1-4
X	US 2,915,537 A (Meade) 01 December 1959 (01.12.1959) entire document, especially col 2, ln 1-25, ln 45-70; col 3, ln 1-15; col 4, ln 1-col 5, ln 7	1-4
A	US 4,792,418 A (Rubin et al.) 20 December 1988 (20.12.1988) entire document, especially col 1, ln 12-22; col 3, ln 33-54; col 5, ln 35-40, ln 53-65; col 7, ln 17-25	1-4
A	US 4,601,856 A (Suzuki et al.) 22 July 1986 (22.07.1986) entire document, especially abstract; col 1, ln 55-68, col 2, ln 1-23, ln 40-45; col 3, ln 15-20, ln 45-55	1-4

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 May 2012 (11.05.2012)	29 MAY 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)