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(54) Title: METHODS AND COMPOSITIONS FOR TREATING NON-ALCOHOLIC FATTY LIVER DISEASE WITH DOCSAHEXAENOIC ACID AND N-ACETYL L-CYSTEINE

(57) Abstract: The present invention is related to methods and compositions for treating non-alcoholic fatty liver disease (NAFLD), and methods of enhancing liver health and/or liver function, in a subject in need thereof, the methods comprising administering to the subject about 200 mg to about 12 g of docosaheptaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and an effective amount of N-acetyl L-cysteine (NAC).



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METHODS AND COMPOSITIONS FOR TREATING NON-ALCOHOLIC FATTY LIVER DISEASE WITH DOCOSAHEXAENOIC ACID AND N-ACETYL L-CYSTEINE

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention is related to methods and compositions for treating non-alcoholic fatty liver disease (NAFLD), and methods of enhancing liver health and/or liver function, in a subject in need thereof, the methods comprising administering about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and an effective amount of N-acetyl L-cysteine (NAC) to the subject in need thereof.

Background

[0002] Nonalcoholic fatty liver disease (NAFLD) refers to a spectrum of liver disease including simple fatty liver (hepatosteatorosis), nonalcoholic steatohepatitis (NASH), and cirrhosis (irreversible, advanced scarring of the liver), that result from accumulation of fat in liver cells, that is not due to excessive alcohol intake. The exact cause of NAFLD is not well understood, but NAFLD is often correlated with other conditions, such as obesity, high cholesterol, and diabetes. Hepatosteatorosis is the accumulation of fat in the liver. Steatohepatitis is characterized by fat accumulation in the liver concurrent with hepatic inflammation. Both alcoholic steatohepatitis, resulting from excessive alcohol intake, and NASH can each lead to cirrhosis and hardening of the liver, resulting in serious liver damage.

[0003] Polyunsaturated fatty acids ("PUFAs," including long-chain PUFAs) have been reported to ameliorate hepatosteatorosis in subjects with non-alcoholic fatty liver disease. Capanni, M. *et al.*, *Aliment. Pharmacol. Ther.*, 23: 1143-1151 (2006). N-acetyl L-cysteine (NAC) has also been reported to attenuate hepatic oxidative stress and decrease fibrosis in a rat model of NASH. Baumgardner, J.N. *et al.*, *J. Nutrition*, 138:1872-9 (2008). NAC is a derivative of the dietary amino acid L-cysteine and is a precursor of the antioxidant glutathione.

[0004] However, there is a need for increasingly effective methods and compositions for the prevention and treatment of NAFLD.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention is directed to a method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA) to the subject in need thereof. Also provided herein is a method of treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering (a) a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA, and (b) an effective amount of N-acetyl L-cysteine (NAC) to the subject in need thereof.

[0006] The present invention is also directed to a method of enhancing liver health and/or function in a subject in need thereof comprising administering a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA to the subject in need thereof. Also provided herein is a method of enhancing liver health and/or function in a subject in need thereof, the method comprising administering (a) a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA, and (b) an effective amount of NAC to the subject in need thereof.

[0007] The present invention is also directed to a composition comprising (a) about 200 mg to about 700 mg of DHA, and (b) about 200 mg to about 700 mg of NAC.

[0008] In some embodiments, the subject has been diagnosed with non-alcoholic steatohepatitis (NASH). In some embodiments, the administration of DHA reduces the incidence and severity of hepatocellular rarefaction and/or hepatocellular vacuolation in the subject. In some embodiments, the subject has an alanine transaminase (ALT) plasma level of greater than about 40 IU/L.

DETAILED DESCRIPTION OF THE INVENTION

[0009] 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.

- [0010] 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.
- [0011] 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.”
- [0012] 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.
- [0013] The present invention is also directed to a method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA) to the subject in need thereof. Also provided herein is a method of treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering (a) a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA, and (b) an effective amount of N-acetyl L-cysteine (NAC) to the subject in need thereof.
- [0014] The present invention is also directed to a method of enhancing liver health and/or function in a subject in need thereof comprising administering a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA to the subject in need thereof. Also provided herein is a method of enhancing liver health and/or function in a subject in need thereof, the method comprising administering (a) a dosage form of about 200 mg to about 12 g of DHA substantially free of EPA, and (b) an effective amount of NAC to the subject in need thereof.
- [0015] The present invention is also directed to a composition comprising (a) about 200 mg to about 700 mg of DHA, and (b) about 200 mg to about 700 mg of NAC.
- [0016] In some embodiments, the subject has been diagnosed with non-alcoholic steatohepatitis (NASH). In some embodiments, the administration of DHA reduces the incidence and severity of hepatocellular rarefaction and/or hepatocellular vacuolation in the subject. In some embodiments, the administration of DHA reduces the severity of hepatic inflammation. In some embodiments, the subject has an alanine transaminase

(ALT) plasma level of greater than about 40 IU/L. In some embodiments, the present invention is directed to a method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and an effective amount of N-acetyl L-cysteine (NAC) to the subject in need thereof.

[0017] The present invention is also directed to a method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and about 200 mg to about 2 g of N-acetyl L-cysteine (NAC) to the subject in need thereof.

[0018] In some embodiments, the present invention is directed to a method of enhancing liver health and/or function in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) in the substantial absence of eicosapentaenoic acid (EPA), and an effective amount of N-acetyl L-cysteine (NAC) to the subject in need thereof.

[0019] The present invention is also directed to a method of enhancing liver health and/or function in a subject in need thereof, the method comprising administering a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and about 200 mg to about 2 g of N-acetyl L-cysteine (NAC) to the subject in need thereof.

[0020] The present invention is also directed to a composition comprising: (a) about 200 mg to about 700 mg docosahexaenoic acid (DHA), and (b) about 200 mg to about 700 mg of N-acetyl L-cysteine (NAC).

[0021] The term "DHA" refers to docosahexaenoic acid, also known by its chemical name (all-Z)-4,7,10,13,16,19-docosahexaenoic acid. DHA is an omega-3 polyunsaturated fatty acid.

[0022] The DHA can be in any form, e.g., triglyceride, diglyceride, monoglyceride, free fatty acid, ester, phospholipid, or combinations thereof. In some embodiments, the DHA of the present invention is 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. Examples of more common esters include C₁-C₆ 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., C₁-C₆ cycloalkyl, or C₁-C₆ aryl ester. 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 a ester. One of skill in the art can appreciate that some non-esterified DHA molecules can be present in the present invention, 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.

[0023] 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 of the present invention 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.

[0024] 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.

[0025] The DHA of the present invention can be derived from various sources, e.g., from oleaginous microorganisms. 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 can be derived from a diatom, e.g., a marine dinoflagellates (algae), such as *Cryptocodinium* sp., *Thraustochytrium* sp., *Schizochytrium* sp., or combinations thereof.

[0026] The source of the DHA can include a microbial source, including the microbial groups Stramenopiles, Thraustochytrids, and Labrinthulids. Stramenopiles include 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 *amoeboides*, *kerguelensis*, *minuta*, *profunda*, *radiata*, *sailens*, *sarkariana*, *schizochytrops*, *visurgensis*, *yorkensis*), *Aplanochytrium* (species include *halitidis*, *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).

[0027] In some embodiments, the algal source is, e.g., *Cryptocodinium cohnii*. Samples of *C. cohnii*, have been deposited with the American Type Culture Collection at Rockville, Md., and assigned accession nos. 40750, 30021, 30334-30348, 30541-30543, 30555-30557, 30571, 30572, 30772-30775, 30812, 40750, 50050-50060, and 50297-50300.

[0028] 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. 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. Cultivation of dinoflagellates such as *C. cohnii* has been described previously. See, U.S. Pat. No. 5,492,938 and Henderson *et al.*, *Phytochemistry* 27:1679-1683 (1988). Organisms useful in the production of DHA can also include any manner of transgenic or other genetically modified organisms, 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.

[0029] In some embodiments, DHA is derived from an animal source. Examples of animal sources include aquatic animals (e.g., fish, marine mammals, crustaceans, rotifers, etc.) and lipids extracted from animal tissues (e.g., brain, liver, eyes, etc.).

[0030] 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 DPA_n6 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, amount of EPA, ARA, DPA_n6, 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.

[0031] 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 condition" by Ellis, incorporated herein by reference.

[0032] 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. 2005/012739; 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 2003/09628; 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 water-soluble from native substance mixtures by centrifugal force," all of which are incorporated herein by reference in their entirety.

[0033] In some embodiments, DHA ester synthesis and purification can include a method comprising: a) reacting purified DHA oil in the presence of an alcohol and a base to produce an ester of a polyunsaturated fatty acid from the triglycerides; and b) distilling the composition to recover a fraction comprising the ester of the polyunsaturated fatty acid, optionally wherein the method further comprises: c) combining the fraction comprising the ester of the polyunsaturated fatty acid with urea in a medium; d) cooling

or concentrating the medium to form a urea-containing precipitate and a liquid fraction; and e) separating the precipitate from the liquid fraction. See, e.g., U.S. Pub. No. 2009/0023808 A1, incorporated by reference herein in its entirety. In some embodiments, the synthesis and purification process of DHA esters includes starting with refined, bleached, and deodorized oil (RBD oil), then performing low temperature fractionation using acetone to provide a concentrate. The concentrate can be obtained by base-catalyzed transesterification, distillation, and silica refining to produce the final DHA product.

[0034] Means of determining purity levels of fatty acids are known in the art, and can include, e.g., chromatographic methods such as HPLC silver ion chromatographic columns (ChromSpher 5 Lipids HPLC Column, Chrompack, Raritan NJ). Alternatively, the purity level can be determined by gas chromatography, with or without converting DHA to the corresponding methyl ester.

[0035] In some embodiments, the DHA esters of the invention can be derived from undiluted oil from a single cell microorganism, and in some embodiments, from undiluted DHASCO-T algal oil (Martek Biosciences Corporation, Columbia, MD). In some embodiments, the oil from which DHA of the invention 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% wt/wt 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.

[0036] Thus, to produce oil from which DHA of the invention can be derived, in certain aspects of the invention, 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.

[0037] 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.

[0038] 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.

[0039] 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.

[0040] 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.

- [0041] 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.
- [0042] DHA free fatty acids can be made using, for example, the DHA containing oils described above. In some embodiments, the DHA-FFA can be obtained from DHA esters. DHA triglycerides, for example, can be saponified followed by a urea adduction step to make free fatty acids.
- [0043] 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 70 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 98wt% of total wt of fatty acid content or at least 99 wt% of total wt of fatty acid content.
- [0044] In some embodiments, DHA is present in an amount of about 35% to about 99.9% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 40% to about 99% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 45% to about 98% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 65% to about 99.9% (wt/wt) of the total fatty acid content of the dosage form or unit dose, or about 85% to about 95% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA is present in an amount greater than about 65% (wt/wt) of the total fatty acid content of the dosage form or unit dose, greater than about 85% (wt/wt) of the total fatty acid content of the dosage form or unit dose, greater

than about 90% (wt/wt) of the total fatty acid content of the dosage form or unit dose, or greater than about 95% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the oil can be diluted with sunflower oil to achieve the desired concentration of fatty acids.

[0045] In some embodiments, the DHA is about 30% (wt/wt) or more of the total fatty acid content of the dosage form or unit dose, about 30% to about 99.9% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 35% to about 99.9% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 35% to about 60% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 35% to about 50% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 37% to about 45% (wt/wt) of the total fatty acid content of the dosage form or unit dose, or about 38% to about 43% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA is greater than about 35%, about 37%, about 38%, about 39% or about 40% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA is about 30% to about 99.5% (wt/wt) of the total fatty acid content of the dosage form or unit dose, or about 40% to about 65% (wt/wt) of the total fatty acid content of the dosage form or unit dose.

[0046] In some of these embodiments, the DHA comprises about 40% to about 45% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some of these embodiments, the DHA comprises about 35% to about 45% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some of embodiments, the DHA comprises about 55% to about 67% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA comprises greater than about 70% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA comprises greater than about 85% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA comprises about 85% to about 99.5% (wt/wt) of the total fatty acid content of the dosage form or unit dose.

[0047] In some embodiments, the DHA is greater than about 80% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 80% to 99.9% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 85% to about 99% (wt/wt) of the total fatty acid content of the dosage form or unit dose, about 87% to about 98% (wt/wt) of the total fatty acid content of the dosage form or unit dose, or about 90% to

about 97% (wt/wt) of the total fatty acid content of the dosage form or unit dose. In some embodiments, the DHA is great than about 95%, about 97%, about 98%, about 99% or about 99.5% (wt/wt) of the total fatty acid content of the dosage form or unit dose.

[0048] In some embodiments, the DHA comprises about 35% to about 96% of the weight of the dosage form or unit dose. In some embodiments, the DHA comprises about 38% to about 42% of the weight of the dosage form or unit dose. In some embodiments, the DHA in the dosage form or unit dose comprises about 35% to about 45% of the total weight of the dosage form or unit dose. In some embodiments, the DHA in the dosage form or unit dose comprises about 55% of the total weight of the dosage form or unit dose. In some embodiments, the DHA in the dosage form or unit dose comprises about 85% to about 96% of the total weight of the dosage form or unit dose.

[0049] In some embodiments, the DHA is about 30% (wt/wt) or more of the total oil content of the dosage form or unit dose, about 30% to about 99.9% (wt/wt) of the total oil content of the dosage form or unit dose, about 35% to about 99.9% (wt/wt) of the total oil content of the dosage form or unit dose, about 35% to about 60% (wt/wt) of the total oil content of the dosage form or unit dose, about 35% to about 50% (wt/wt) of the total oil content of the dosage form or unit dose, about 37% to about 45% (wt/wt) of the total oil content of the dosage form or unit dose, or about 38% to about 43% (wt/wt) of the total oil content of the dosage form or unit dose. In some embodiments, the DHA is greater than about 35%, about 37%, about 38%, about 39% or about 40% (wt/wt) of the total oil content of the dosage form or unit dose. In some embodiments, the DHA is about 30% to about 99.5% (wt/wt) of the total oil content of the dosage form or unit dose, or about 40% to about 65% (wt/wt) of the total oil content of the dosage form or unit dose.

[0050] In some of these embodiments, the DHA comprises about 40% to about 45% (wt/wt) of the total oil content of the dosage form or unit dose. In some of these embodiments, the DHA comprises about 35% to about 45% (wt/wt) of the total oil content of the dosage form or unit dose. In some of embodiments, the DHA comprises about 55% to about 67% (wt/wt) of the total oil content of the dosage form or unit dose. In some embodiments, the DHA comprises greater than about 70% (wt/wt) of the total oil content of the dosage form or unit dose. In some embodiments, the DHA comprises about 85% to about 99.5% (wt/wt) of the total oil content of the dosage form or unit dose.

[0051] In some embodiments, the DHA is greater than about 80% (wt/wt) of the total oil content of the dosage form or unit dose, about 80% to 99.9% (wt/wt) of the total oil content of the dosage form or unit dose, about 85% to about 99% (wt/wt) of the total oil content of the dosage form or unit dose, about 87% to about 98% (wt/wt) of the total oil content of the dosage form or unit dose, or about 90% to about 97% (wt/wt) of the total oil content of the dosage form or unit dose. In some embodiments, the DHA is greater than about 95%, about 97%, about 98%, about 99% or about 99.5% (wt/wt) of the total oil content of the dosage form or unit dose. 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.

[0052] 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.

[0053] As noted above, in the embodiments herein, DHA is administered substantially free of eicosapentaenoic acid (EPA). 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 ω -3 polyunsaturated fatty acid. DHA is "substantially free of EPA" when EPA is less than about 3% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, EPA comprises less than about 2% (wt/wt) of the total fatty acid content of the dosage form, less than 1% (wt/wt) of the total fatty acid content of the dosage form, less than 0.5% (wt/wt) of the total fatty acid content of the dosage form, less than 0.2% (wt/wt) of the total fatty acid content of the dosage form, or less than 0.01% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, the dosage form has no detectable amount of EPA.

[0054] DHA can also be administered substantially free of arachidonic acid (ARA). 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 an ω -6 polyunsaturated fatty acid.

DHA is "substantially free of ARA" when ARA is less than about 3% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, ARA comprises less than about 2% (wt/wt) of the total fatty acid content of the dosage form, less than 1% (wt/wt) of the total fatty acid content of the dosage form, less than 0.5% (wt/wt) of the total fatty acid content of the dosage form, less than 0.2% (wt/wt) of the total fatty acid content of the dosage form, or less than 0.01% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, the dosage form has no detectable amount of ARA.

[0055] DHA can also be administered substantially free of docosapentaenoic acid 22:5n-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 an ω -6 polyunsaturated fatty acid. DHA is "substantially free of DPAn6" when DPAn6 is less than about 3% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, DPAn6 comprises less than about 2% (wt/wt) of the total fatty acid content of the dosage form, less than 1% (wt/wt) of the total fatty acid content of the dosage form, less than 0.5% (wt/wt) of the total fatty acid content of the dosage form, less than 0.2% (wt/wt) of the total fatty acid content of the dosage form, or less than 0.01% (wt/wt) of the total fatty acid content of the dosage form. In some embodiments, the dosage form has no detectable amount of DPAn6.

[0056] 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; croetnoids; 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.

[0057] 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).

[0058] In the methods and compositions herein, additional fatty acids can be present in the dosage form or unit dose or composition. These fatty acids can include fatty acids that were not removed during the purification process, i.e., fatty acids that were co-isolated with DHA from an organism. In some embodiments, one or more non-DHA fatty acids can be added to the dosage form or unit dose 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 dosage form or unit dose comprises 0.01% to about 4% (wt/wt) of oleic acid. In some embodiments, the dosage form or unit dose comprises 0.01% to 0.5% (wt/wt) 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, a dosage form or unit dose comprises 0.01% to 0.1% (wt/wt) 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, a dosage form or unit dose comprises less than 0.5% (wt/wt) 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 dosage form or unit doses 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).

[0059] In some embodiments, the dosage form or unit dose comprises 0.1% to 60% (wt/wt) 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 dosage form or unit dose

comprises 20% to 40% (wt/wt) 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 dosage form or unit dose comprises less than 1% (wt/wt) 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).

[0060] In some embodiments, the dosage form or unit dose is characterized by a fatty acid content of about 0.1% to about 20% (wt/wt) 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).

[0061] In some embodiments, a dosage form or unit dose is characterized by a fatty acid content of about 1.0% to about 5% (wt/wt) 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 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8).

[0062] In some embodiments, the dosage form 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).

[0063] In some of embodiments of DHA dosage form 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 (wt/wt) of capric acid (C10:0). The embodiments herein may further comprise about 6% or less (wt/wt) of lauric acid (C12:0). The embodiments herein may further comprise about 20% or less (wt/wt), or about 5% to about 20% (wt/wt) of myristic acid (C14:0). The embodiments herein may further comprise about 20% (wt/wt) or less, or about 5% to about 20% (wt/wt)

of palmitic acid (C16:0). The embodiments herein may further comprise about 3% (wt/wt) or less of palmitoleic acid (C16:1n-7). The embodiments herein may further comprise about 2% (wt/wt) or less of stearic acid (C18:0). The embodiments herein may further comprise about 40% (wt/wt) or less, or about 10% to about 40% (wt/wt) of oleic acid (C18:1n-9). The embodiments herein may further comprise about 5% (wt/wt) or less of linoleic acid (C18:2). The embodiments herein may further comprise about 2% (wt/wt) or less of nervonic acid (C24:1). The embodiments herein may further comprise about 3% (wt/wt) 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).

[0064] In some embodiments, the dosage form comprises, measured in percentage of free fatty acid, about 35-65%, 40-55%, 35-57%, or 57-65% DHA (22:6 n-3); about 0-2% capric acid (10:0); about 0-6% lauric acid (12:0); about 10-20% myristic acid (14:0); about 5-15% palmitic acid (16:0); about 0-5% palmitoleic acid (16:1); about 0-2% stearic acid (18:0); about 5-20% or 5-25% oleic acid (18:1); about 0-2% linoleic acid (18:2); and about 0-2% 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%

[0065] The present invention can also provide 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, 65 wt. % DHA, optionally in triglyceride form, as a percentage of total fatty acids.

[0066] 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 (wt/wt)
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% to 45%
24:1	Max 2%
Others	Max 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 0.04 ppm
Phosphorous	Max 10 ppm
Chemical Characteristics	
Peroxide value	Max 5 meq/kg
Free fatty acid	Max 0.4%
Unsaponifiable Matter	Max 3.5%

[0067] An exemplary undiluted DHA (triglyceride) containing oil derived from *Cryptocodinium 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

Test	Specification
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%

[0068] 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).

[0069] 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 Acids	Concentration (wt/wt)
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%

[0070] In some embodiments and 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 docosapentaenoic 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 tetracosaoenoic acid (24:1n9). The embodiments provided herein may further comprise about 1% or less (w/w) of other fatty acids. The DHA in 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 *Cryptocodinium*, *cohnii* sp.

[0071] In some embodiments, the DHA composition may comprise DHASCO[®]. DHASCO[®] is an oil derived from *Cryptocodinium 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%.

[0072] 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, 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, 103,

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.

[0073] 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 DPA (n-3) in the compositions may be at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 wt. % of DPA (n-3). The DPA (n-3) may be present in triglyceride or ester form. For example, the DPA (n-3) may be present in ethyl ester form. In certain embodiments, the compositions comprise all three of the DHA, C28:8 and DPA (n-3) in the concentration ranges specified above.

[0074] In further embodiments, the compositions may comprise less than about 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 wt. % EPA in addition to the DHA and C28:8. In one embodiment, the compositions may comprise less than about 0.25 wt. % EPA. The EPA may be present in any form, including triglyceride or ester form. In some embodiments, the compositions may comprise 0 wt. % EPA.

[0075] The present invention also provides compositions comprising at least about 90 wt. % of DHA and at least one additional fatty acid or a derivative thereof. In some embodiments, the amount of DHA in the compositions may be at least about 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt. %. In certain embodiments, the additional fatty acid may have a boiling point of about 150-170°C at a pressure of 0.8 mm Hg.

[0076] An exemplary DHA-containing oil derived from the algal oil of *Cryptocodinium cohnii*, wherein the DHA comprises an ethyl ester, can be characterized by the specified amount of components listed in **Table 4**, where "Max" refers to the amount of the component that can be present up to the specified amount.

Table 4

DHA content (mg/g)	855-945
Fatty Acid Content: % of total EE	
Eicosapentaenoic Acid (20:5 ω 3)	ND
Myristic Acid (14:0)	0.1%
Palmitic Acid (16:0)	0.5%
Palmitoleic Acid (16:1 ω 7)	0.4%
Stearic Acid (18:0)	ND
Oleic Acid (18:1 ω 9)	4%
Linoleic Acid (18:2 ω 6)	ND
Docosapentaenoic acid (22:5 ω 3)	1.3%
Octacosaoctanoic acid (28:8 ω 3)	0.9%
Tetracosanoic Acid (24:1 ω 9)	0.3%
Others	1.1%
Elemental Composition	
Arsenic	Max 0.5 ppm
Copper	Max 0.1 ppm
Iron	Max 0.5 ppm
Lead	Max 0.2 ppm
Mercury	Max 0.04 ppm
Chemical Characteristics	
Peroxide value	Max 10.0 meq/kg

ND = not detectable

[0077] In some embodiments of the 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 docosapentaenoic 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).

[0078] In some embodiments, more specifically, DHA-S (Martek Biosciences, Columbia, MD) 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%; eicosapentaenoic 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%.

[0079] In some embodiments, the dosage form 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% 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*.

[0080] 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 5

Fatty Acids	Concentration (wt/wt)
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 EPA	Max 3%
22:5n-6 DPA	12%-18%
22:6 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%

[0081] 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).

[0082] 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.

- [0083] 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.
- [0084] The DHA in an oil 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 derived from the Thraustochytrid, *Schizochytrium sp.* An exemplary DHA (ethyl esters) containing oil derived from *Schizochytrium sp.* is characterized by the specified amount of components listed in Table 4 of WO 2009/006317, incorporated by reference herein. In some of these embodiments, an oil comprises DHA $\geq$ than about 57% (w/w), particularly $\geq$ about 70% (w/w) of the total fatty acid content of the oil or unit dose. In some of these embodiments, the ratio of wt% of DHA to wt% of DPAn6 is about 2.5 to about 2.7.
- [0085] In some embodiments, the composition or oil is characterized by one or more 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.*

[0086] An exemplary DHA (free fatty acid) containing oil is characterized by the specified amount of components listed in **Table 6**:

Table 6

Fatty Acids	Concentration (wt/wt)
10:0	Max 0.5%
12:0	Max 0.5%
14:0	Max 0.5%
14:1	Max 0.5%
16:0	Max 0.5%
16:1	Max 0.5%
18:1 (n-9)	Max 0.5%
20:5 (n-3) EPA	Max 0.5%
22:5 (n-3) DPA	Max 1%
22:6 (n-3) DHA	Min 95%
28:8	Max 1.5%
Chemical Characteristics	
Docosahexaenoic acid	946 mg/g
Docosahexaenoic acid	98%
Free Fatty Acids	93%
Trans Fatty Acids	<1%

[0087] 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).

[0088] 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.

- [0089] In some embodiments, the composition of DHA may further comprise vitamin E. Compounds of the vitamin E group are fat-soluble vitamins with antioxidant properties and include eight related α -, β -, γ -, and δ -tocopherols and the corresponding four tocotrienols. In some embodiments, the vitamin E in the composition is a tocopherol. In some embodiments, the tocopherol is selected from α -, β -, γ -, and δ -tocopherols, or combinations thereof.
- [0090] N-acetyl-L-cysteine (NAC) is the N-acetyl derivative of the natural amino acid L-cysteine. NAC, or acetylcysteine (ACC), refers to (R)-2-acetamido-3-sulfanypropanoic acid (CAS No. 616-91-1), and isomers, racemates, and salts thereof.
- [0091] NAC is commercially available in a variety of different solid forms, with varying average particle size from less than about 50 microns to over about 600 microns, and all of these forms can be used in the present invention. See, e.g., U.S. Patent No. 6,623,754. NAC can also be manufactured according to known methods. For example, NAC can be generated enzymatically, by subjecting L-2-oxothiazolidine-4-carboxylate to the action of 5-oxo-L-prolinase in the presence of adenosinetriphosphate. See, e.g., U.S. Patent No. 4,335,210. In addition, NAC can also be generated electrochemically, by acetylation of L-cystine to produce N-acetyl-cystine, followed by electroreduction and desalination by electrodialysis of the N-acetyl-cystine to produce NAC. See, e.g., U.S. Patent No. 6,159,352.
- [0092] The present invention can be directed to methods of treating non-alcoholic fatty liver disease (NAFLD). "Hepatosteatorosis" refers to the accumulation of fat in the liver cells. "NAFLD" refers to the accumulation of fat in the liver cells ("fatty liver") that is not due to excessive alcohol use. As used herein, "non-alcoholic steatohepatitis," or "NASH," refers to a type of NAFLD, wherein hepatosteatorosis is accompanied by hepatic inflammation. "Inflammation" refers to a fundamental pathological processes consisting of a dynamic complex of cytological and chemical reactions in response to an injury or abnormal stimulation caused by a physical, chemical or biological agent. NASH can lead to liver fibrosis and cirrhosis. "Fibrosis" refers to the development of excess fibrous connective tissue in a tissue or organ as a consequence of a reparative or reactive process, as opposed to the development of fibrous tissue as a normal constituent of an organ or tissue. Liver fibrosis is also referred to as hardening of the liver. "Cirrhosis" refers to a liver disease in which damaged normal liver cells are replaced by scar tissue, decreasing

the amount of normal or functioning liver tissue. NASH can also lead to increased hepatocellular rarefaction and hepatocellular vacuolation. "Hepatocellular rarefaction" refers to a decrease in the cytoplasmic density of hepatocytes. "Hepatocellular vacuolation" refers to the formation of vacuoles within hepatocytes.

[0093] Animal models used for studying NAFLD and NASH are known in the art. For example, rodents with genetic defects, such as leptin deficiency, have been used. See, e.g., Matsusue, K. *et al.*, *Cell Metab.*, 7: 302–311 (2008). NASH has been surgically induced by bile duct ligation. See, e.g., Lee, S. *et al.*, *J Pediatr. Surg.*, 43:2010-5 (2008). NAFLD has been observed in subjects whose diet lacked essential fatty acids or nutrients. See, e.g., Puder, M. *et al.*, *Ann. Surg.*, 250:395-402 (2009). Liver fibrosis has been induced chemically. See, e.g., Gonzalez-Periz, A. *et al.*, *FASEB J.*, 20:2537-9 (2006); Fernandez, I. *et al.*, *Exp. Toxicol. Pathol.*, 57:65-75 (2005). NASH has been induced by administration of a diet designed to produce insulin resistance, steatosis, inflammation, and fibrosis. See, e.g., Svegliati-Baroni, G. *et al.*, *Am. J. Pathol.*, 169:846-60 (2006); Carmiel-Haggai, M. *et al.*, *FASEB J.*, 19:136-8 (2005). Each of these references are incorporated herewith in their entirety.

[0094] Subjects suffering from NAFLD or NASH can display certain symptoms and/or findings such as, fatty liver deposits, liver tissue degeneration, liver inflammation, liver cell degeneration, liver cirrhosis, elevated free fatty acids, elevated aspartate transaminase (AST) and/or alanine transaminase (ALT) activity, and/or other such abnormalities. A "transaminase" is also referred to by the term "aminotransferase."

[0095] Biomarkers for NAFLD can include serum levels of ALT, AST, alkaline phosphatase (AP), glutamyl transpeptidase (GGT), total bilirubin (TB), and total cholesterol (TC). These biomarker levels can be determined by methods known to one of skill in the art. For example, enzyme immunoassays can be used to measure levels of ALT, AST, AP, and GGT. TB can be measured by employing the Jendrassik-Grof method, wherein bilirubin is reacted with diazotized sulfanilic acid. TC can be measured by colorimetric or spectrophotometric analysis. Thus, in some embodiments, the method is directed to a subject having abnormal levels of one or more biomarkers indicated herein.

[0096] NAFLD or NASH can be diagnosed by liver biopsy. Indications found upon pathological analysis of a liver biopsy that can indicate NAFLD include steatosis,

hepatocellular ballooning, lobular inflammation (necroinflammation), fibrosis, and hepatocyte necrosis. These indications can be studied pathologically by methods known in the art. For example, these indications can be studied using hematoxylin and eosin staining and Masson's trichrome staining. See, e.g., Kleiner, D.E. *et al.*, *Hepatology*, 41: 1313-1321 (2005). Thus, in some embodiments, the method is directed to a subject having one or more indications identified by liver biopsy identified herein.

[0097] In some embodiments, the subject of the present invention has alanine transaminase (ALT) plasma levels greater than about 40 IU/L, greater than about 45 IU/L, greater than about 50 IU/L, greater than about 60 IU/L or greater than about 80 IU/L. In some embodiments of the present invention, the DHA and NAC provide a decrease in ALT plasma levels in a subject, e.g., a 10%, 20%, 40% or 50% decrease in ALT plasma levels relative to a subject who is not suffering from NAFLD and/or NASH.

[0098] The method of the present invention can be administered to individuals who have NAFLD or individuals who are at risk for developing NASH. Thus, in some embodiments the invention is directed to a method of treating a subject having NAFLD, or simple hepatosteatorosis, the method comprising administering the DHA and NAC as described herein. The invention is also directed to a method of treating a subject having NASH, or hepatosteatorosis concurrently with hepatic inflammation. The invention is also directed to a method of reducing the incidence and severity of hepatocellular rarefaction and hepatocellular vacuolation.

[0099] The invention can also be directed to a method of enhancing liver health and/or liver function in a subject in need thereof. Indications of enhanced liver health and/or function can include decreased levels of hepatosteatorosis, decreased levels of hepatic inflammation, decreased serum levels of ALT, AST, AP, and GGT, and lower levels of TB and TC.

[00100] The terms "treat" and "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological condition, disorder or disease, or obtain beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms associated with NAFLD and/or NASH; prevention of NAFLD and/or NASH; delay in the onset of NAFLD and/or NASH; reduced incidence of NAFLD and/or NASH in a population; diminishment of the

extent of the condition associated with NAFLD and/or NASH; stabilization (i.e., not worsening) of the state of the condition, disorder or disease associated with NAFLD and/or NASH; delay in onset or slowing of the condition, disorder or disease progression associated with NAFLD and/or NASH; amelioration of the condition, disorder or disease state, remission (whether partial or total) of the condition, disorder or disease associated with NAFLD and/or NASH, whether detectable or undetectable; or enhancement or improvement of the condition, disorder or disease associated with NAFLD and/or NASH. The term "preventing" means to stop or hinder a disease, disorder, or symptom of a disease or condition. Treatment includes eliciting a clinically significant response, without excessive levels of side effects. Treatment also includes prolonging survival as compared to expected survival if not receiving treatment.

[00101] The term "subject" refers to mammals such as humans or primates, such as apes, monkeys, orangutans, baboons, gibbons, and chimpanzees. The term "subject" can also refer to companion animals, e.g., dogs and cats; zoo animals; equids, e.g., horses; food animals, e.g., cows, pigs, and sheep; and disease model animals, e.g., rabbits, mice, and rats. The subject can be a human or non-human. The subject can be of any age. For example, in some embodiments, the subject is a human infant, i.e., post natal to about 1 year old; a human child, i.e., a human between about 1 year old and 12 years old; a pubertal human, i.e., a human between about 12 years old and 18 years old; or an adult human, i.e., a human older than about 18 years old. In some embodiments, the subject is an adult, either male or female. In some embodiments, the subject is a patient.

[00102] In some embodiments, the subject is a "subject in need thereof." A subject in need thereof refers to an individual who has e.g., hepatosteatorosis, hepatic inflammation, cirrhosis, biliary obstruction, and/or hepatic fibrosis. In some embodiments, it is desirable to treat, e.g., to reduce hepatosteatorosis, hepatic inflammation, cirrhosis, biliary obstruction, and/or hepatic fibrosis; prevent hepatosteatorosis, hepatic inflammation, cirrhosis, biliary obstruction, and/or hepatic fibrosis; or retard the onset of hepatosteatorosis, hepatic inflammation, cirrhosis, biliary obstruction, and/or hepatic fibrosis.

[00103] In some embodiments the administration of DHA and NAC can be used to treat hepatic fibrosis. In some embodiments, administration of DHA and NAC can be used to prevent formation of new fibroids. In some embodiments, administration of DHA

and NAC can be used to reduce the number of fibroids. In some embodiments, administration of DHA and NAC can be used to retard the onset of fibroid formation.

[00104] In the course of examination of a subject, a medical professional can determine that administration of one of the methods of the present invention is appropriate for the subject, or the physician can determine that the subject's condition can be improved by the administration of one of the methods of the present invention. Prior to prescribing any DHA/NAC regimen, the physician can counsel the subject, for example, on the various risks and benefits associated with the regimen. The subject can be provided full disclosure of all the known and suspected risks associated with the regimen. Such counseling can be provided verbally, as well as in written form. In some embodiments, the physician can provide the subject with literature materials on the regimen, such as product information, educational materials, and the like.

[00105] A "medical professional," includes, for example, a physician, physician assistant, nurse practitioner, pharmacist and customer service representative. All of the various aspects, embodiments and options described herein can be combined in any and all variations.

[00106] Administration of DHA and NAC can be combined with other regimens (i.e., non-DHA/NAC regimens) used to treat NAFLD and/or NASH. For example, the method of the present invention can be combined with diet regimens (e.g., low carbohydrate diets, high protein diets, high fiber diets, etc.), exercise regimens, or weight loss regimens, to treat NAFLD and/or NASH. The methods of the present invention can also be used in combination with other pharmaceutical products to treat NAFLD and/or NASH in a subject.

[00107] In some embodiments, the DHA and NAC of the present invention are administered before the non-DHA/NAC regimens. Alternatively, in some embodiments, the non-DHA/NAC regimens are administered first, and then the DHA and NAC of the present invention are administered. In some embodiments, the DHA and NAC regimen and the non-DHA/NAC regimen are administered at the same time.

[00108] In some embodiments, the DHA and/or NAC is administered in a dosage form. As defined herein, "dosage form" refers to the physical form of an administration dosage. The term "dosage form" can refer to any traditionally used or accepted administrative forms, such as oral administrative forms, intravenous administrative forms, or

intraperitoneal administrative forms. As used herein, the term "dosage form" refers to an amount of DHA and/or NAC administered to a subject in a unit dose, e.g., in a gel capsule. As used herein, a unit dose is a single unit of administration, e.g., a single dosage form. Alternatively, the term "dosage form" refers to an amount of DHA that is administered to a subject at a single time period, e.g., two pills swallowed at the same time, or one pill swallowed shortly after another pill. The term "dosage form" can also refer to a unit of solid (e.g., capsules, caplets, tablets, or pills), liquid, syrup, beverage, or food item, that is swallowed within a short period of time, e.g., within 1 minute, 2 minutes, 3 minutes, 5 minutes, 10 minutes, 20 minutes or 30 minutes. In some embodiments, the term "dosage form" can also refer to unformulated oils.

[00109] In some embodiments, the DHA is administered in a single dosage form, i.e., a unit dose. As used herein, a "unit dose" refers to an amount of DHA administered to a subject in a single dosage form, e.g., in a gel capsule. The term "unit dose" can also refer to a unit of pharmaceutically suitable solid, liquid, syrup, beverage, or food item that is swallowed within a short period of time, e.g., within 1 minute, 2 minutes, 3 minutes, 5 minutes, 10 minutes, 20 minutes, or 30 minutes.

[00110] In some embodiments, the dosage form is and components thereof are pharmaceutically acceptable. "Pharmaceutically acceptable" refers to compositions that are, within the scope of sound medical judgment, suitable for contact with the tissues of human beings and animals without excessive toxicity or other complications commensurate with a reasonable benefit/risk ratio. In some embodiments, the compounds (e.g., DHA), compositions, and dosage forms of the present invention are pharmaceutically acceptable.

[00111] In some embodiments, the dosage form is a nutraceutical dosage form. The term "nutraceutical" refers to any substance that is (1) a sole item of a meal or diet that provides medical and/or health benefits, or (2) a product that is intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients that provides medical and/or health benefits. The medical and/or health benefits can include reducing the risk of a condition by decreasing hepatosteatosis and/or hepatic inflammation.

[00112] In some embodiments, the DHA and/or NAC may be provided in a dietary supplement, medical food or animal feed. "Dietary supplement" refers to a compound or composition used to supplement the diet of an animal or human. In some embodiments, the dietary supplement can further comprise various "dietary ingredients" intended to supplement the diet. "Dietary ingredients" can further include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary ingredients can also include extracts or concentrates. In some embodiments, the dosage form of DHA is administered in a dietary supplement. In some embodiments, about 200 mg to about 2g of DHA per day is administered in a dietary supplement. In some embodiments, the NAC is administered in a dietary supplement. In some embodiments, about 200 mg to about 2 g per day of NAC is administered in a dietary supplement.

[00113] The total weight of the dosage form or unit dose can vary. In some embodiments, the dosage form or unit dose has a total weight of about 200 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 550 mg, about 600 mg, about 650 mg, about 700 mg, about 750 mg, about 800 mg, about 850 mg, about 900 mg, about 950 mg, about 1.0 g, or about 1.05 g.

[00114] In some embodiments, the dosage form or unit dose comprises about 0.2 g to about 1 g of DHA. In some embodiments, the dosage form or unit dose comprises about 200 mg, about 450 mg, about 900 mg, about 1.35 g, about 1.8 g, about 2.25 g, about 2.7 g, about 3.15 g, about 3.6 g, about 4.05 g, about 4.5 g, about 4.95 g, about 5.4 g, about 5.85 g, about 6.3 g, about 6.75 g, about 7.2 g, about 7.65 g, about 8.1 g, about 8.55 g, about 9 g, about 9.45 g, about 9.9 g, about 10.35 g, about 10.8 g, about 11.25 g, about 11.7 g, or about 12 g DHA. In some embodiments, the dosage form or unit dose comprises about 200 mg to about 1.8 g, about 200 mg to about 1.35 g, or about 900 mg. In some embodiments, the dosage form or unit dose comprises about 855 mg to about 945 mg or about 873 mg to about 927 mg DHA.

[00115] As used herein, "composition" refers to a combination of two or more materials, substances, excipients, portions, and the like. In some embodiments, the composition comprises about 200 mg to about 700 mg DHA, about 300 mg to about 600 mg DHA, about 400 mg to about 500 mg DHA, or about 450 mg DHA, and about 200 mg to about

500 mg NAC, or about 250 to about 400 mg NAC, or about 300 mg to about 350 mg NAC.

[00116] The term "administering" or "administration" of the composition refers to the application of the composition, e.g., oral or parenteral (e.g., transmucosal, intravenous, intramuscular, subcutaneous, rectal, intravaginal, or via inhalation) to the subject. Administering would also include the act of prescribing a composition described herein to a subject by a medical professional for treatment of NAFLD and/or NASH. Administering can also include the act of labeling a composition, i.e., instructing a subject to administer a composition, in a manner as provided herein for treatment of NAFLD and/or NASH.

[00117] Various dosage amounts of DHA and NAC can be administered to a subject. The terms "daily dosage," "daily dosage level," and "daily dosage amount" refer to the total amount of DHA and/or NAC administered per day (about a 24 hour period). Thus, for example, administration of DHA to a subject at a daily dosage of about 900 mg means that the subject receives a total of about 900 mg of DHA in a 24 hour period, whether the DHA is administered as a single dosage form or unit dose comprising about 900 mg DHA, or alternatively, two dosage forms or unit doses comprising about 450 mg DHA each (for a total of about 900 mg DHA).

[00118] In some embodiments, the daily amount of DHA is administered in a single dosage form, or in two or more dosage forms. In some embodiments, the daily amount of DHA is administered in three dosage forms, four dosage forms, five dosage forms, or six or more dosage forms. The dosage forms of the present invention can be taken in a single application or multiple applications per day. For example, if four dosage forms are taken daily, then all four dosage forms can be taken once daily, or 2 dosage forms can be taken twice daily, or 1 dosage form can be taken every 6 hours, or each of the 4 dosage forms can be taken at various differential time points within a twenty four hour period.

[00119] In some embodiments, the DHA is administered in an amount of from about 1.5 mg per kg body weight per day to about 125 mg per kg body weight per day. In some embodiments, the DHA is administered in an amount of from about 150 mg to about 10 g per day; from about 0.5 g per day to about 5 g per day; or from about 1 g per day to about 5 g per day.

[00120] In some embodiments, the daily amount of DHA is about 200 mg, about 900 mg, about 1350 mg, about 1800 mg, about 2250 mg, about 2700 mg, about 3150 mg, about 3600 mg, about 4050 mg, about 4500 mg, about 4950 mg, about 5400 mg, about 5850 mg, about 6300 mg, about 6750 mg, about 7200 mg, about 7650 mg, about 8100 mg, about 8550 mg, about 9000 mg, about 9450 mg, about 9900 mg, about 10.35 g, about 10.8 g, about 11.25 g, about 11.7 g, or about 12 g DHA. In some embodiments, the daily amount of DHA is about 200 mg to about 12 g, about 900 mg to about 5400 mg, about 1800 mg to about 4500 mg, or about 2700 mg to about 3600 mg. In some embodiments, about 0.84 g to about 4 g of DHA is administered per day to the subject. In some embodiments, about 0.84 g to about 1.5 g of DHA is administered per day to the subject. In some embodiments, about 0.84 mg to about 1.0 g of DHA is administered per day to the subject.

[00121] In some embodiments, the dosage form or unit dose comprises about 430 mg to about 480 mg of DHA ethyl ester. In some embodiments, the dosage form or unit dose comprises about 860 mg to about 950 mg of the DHA ethyl ester. In some embodiments, the dosage form or unit dose comprises about 870 mg to about 930 mg of the DHA ethyl ester.

[00122] In some embodiments, the daily dose is provided as a unit dose.

[00123] Various amounts of NAC can be used in the present invention. An "effective amount" of NAC refers to an amount of NAC that is effective in producing the desired result of the present invention, i.e. treating NAFLD, NASH, and/or enhancing liver function or health in a subject in need thereof. In some embodiments, the method of the present invention comprises administering about 200 mg to about 2 g of NAC, about 300 mg to about 1200 mg NAC, about 400 mg to about 1000 mg NAC, about 500 mg to about 800 mg NAC, or about 600 mg to about 700 mg NAC.

[00124] In some embodiments, the method of the present invention comprises simultaneous co-administration of DHA and NAC. Simultaneous co-administration of DHA and NAC refers to substantially contemporaneous administration. Substantially contemporaneously administration of DHA and NAC refers to administration of NAC to a subject before/after one hour of administration of DHA, or administration of NAC to a subject within 30 minutes before/after administration of DHA. In some embodiments, NAC is administered within 20 minutes, within 10 minutes or within 5 minutes

before/after administration of DHA. In some embodiments, NAC is provided within the same dosage form or unit dose as DHA, and thus NAC and DHA are co-administered at the same time.

[00125] Administration of the DHA and NAC of the present invention can be achieved using various regimens. For example, in some embodiments, the DHA and NAC is administered daily on consecutive days, or alternatively, the DHA and/or NAC is administered every other day (bi-daily). Administration can occur on one or more days. In some embodiments, administration of the DHA and NAC continues even after the symptoms of NAFLD and/or NASH have been alleviated. In some embodiments, the administration of the DHA and NAC is administered as a prophylactic measure, before the onset of symptoms associated with NAFLD and/or NASH.

[00126] In some embodiments, the dosage form of DHA and NAC are administered for one day, i.e., a single administration. In some embodiments, the dosage form is administered daily for the remainder of the subject's lifetime. In some embodiments, the dosage form is administered daily for 1 to 10 years. In some embodiments, the dosage form is administered daily for 1 to 12 consecutive months. In some embodiments, the dosage form is administered daily for at least 6 consecutive months. In some embodiments, the dosage form is administered once per day. In some embodiments, the DHA is administered in a single dosage form.

[00127] The compositions of the present invention can be administered for a long duration of time or a short duration of time. For example, in some embodiments, the subject has a chronic condition, and is administered the DHA and NAC of the present invention for more than 6 months, more than one year, more than 2 years, more than 5 years, more than 10 years, or more than 20 years. In some embodiments, the DHA and NAC of the present invention is administered daily for a shorter duration, e.g., 1 week to 12 weeks, or 2 weeks to 6 weeks..

[00128] In some embodiments, the DHA and NAC is administered continuously. The term "continuous" or "consecutive," as used herein in reference to "administration," means that the frequency of administration is at least once daily. Note, however, that the frequency of administration can be greater than once daily and still be "continuous" or "consecutive," e.g., twice or even three or four times daily, as long as the dosage levels as specified herein are not exceeded.

[00129] The DHA and/or NAC can each be formulated in a separate dosage form or they can be formulated together in a single dosage form. These dosage forms can include, but are not limited to, tablets, capsules, caplets, dragees, cachets, pellets, pills, gel caps, powders and granules, syrups, slurries, emulsions, suspensions; and parenteral dosage forms which include, but are not limited to, solutions, suspensions, emulsions, coated particles, and dry powder comprising an effective amount of the DHA and/or NAC as taught in this invention. In some embodiments, dosage form is an intravenous formulation. In some embodiments, the dosage form is a tablet, gel cap, pill or caplet. In some embodiments, the dosage form is a capsule, wherein the capsule is filled with a solution, suspension, or emulsion comprising the DHA and/or the NAC. In some embodiments, the capsule is made out of gelatin (gel cap). It is also known in the art that the active ingredients can be contained in such formulations with pharmaceutically acceptable excipients such as diluents, fillers, disintegrants, binders, lubricants, surfactants, hydrophobic vehicles, water soluble vehicles, emulsifiers, buffers, humectants, moisturizers, solubilizers, preservatives, flavorants, taste-masking agents, sweeteners, and the like. Suitable excipients can include, e.g., vegetable oils (e.g., corn, soy, safflower, sunflower, or canola oil). In some embodiments, the preservative can be an antioxidant, e.g., sodium sulfite, potassium sulfite, metabisulfite, bisulfites, thiosulfates, thioglycerol, thiosorbitol, cysteine hydrochloride, α -tocopherol, tocotrienols, and combinations thereof. The means and methods for administration are known in the art and an artisan can refer to various pharmacologic references for guidance. For example, "Modern Pharmaceutics," Banker & Rhodes, Informa Healthcare, 4th ed. (2002); and "Goodman & Gilman's The Pharmaceutical Basis of Therapeutics," McGraw-Hill, New York, 10th ed. (2001) can be consulted.

[00130] The DHA and NAC of the methods and composition of the present invention are orally active and this route of administration can be used in the invention.

[00131] For oral administration, the DHA can be administered as an oil or it can be formulated readily by combining it with a pharmaceutically acceptable carrier or with pharmaceutically acceptable carriers. Pharmaceutical acceptable carriers are well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, gel caps, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. In some embodiments, the dosage

form is a tablet, gel cap, pill or caplet. Pharmaceutical preparations for oral use can be obtained by adding a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include, but are not limited to, fillers such as sugars, including, but not limited to, lactose, sucrose, mannitol, and sorbitol; cellulose preparations such as, but not limited to, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxymethyl cellulose, vegetable oil (e.g., soybean oil), and polyvinylpyrrolidone (PVP). If desired, disintegrating agents can be added, such as, but not limited to, the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Pharmaceutical preparations which can be used orally include, but are not limited to, hard or soft gelatin capsules. In some embodiments, the encapsulating material comprises a gelatin, a plasticizer, and water. In certain embodiments, the encapsulating material is vegetarian, i.e., made from non-animal derived material, including plants, seaweed (for example, carrageenan), food starch, modified corn starch, potato starch, and tapioca. In other embodiments, the encapsulating material is derived from animals, including porcine, bovine, and fish-based materials, such as gelatins. Plasticizers of the invention include glycerin, glycerol, polyols, and mixtures thereof. In some embodiments, the plasticizer is a high boiling point polyol, such as glycerol or sorbitol.

[00132] It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention can include other suitable agents such as flavoring agents, preservatives, and antioxidants. In particular, it is desirable to mix the DHA with an antioxidant to prevent oxidation. Such antioxidants are pharmaceutically acceptable and can include vitamin E, carotene, BHT or other antioxidants known to those of skill in the art.

[00133] The term "administering" or "administration" of the composition refers to the application of the composition, e.g., oral or parenteral (e.g., transmucosal, intravenous, intramuscular, subcutaneous, rectal, intravaginal, or via inhalation). Administering could also include the act of prescribing a composition described herein to a subject by a medical professional for treatment of NASH. Administering can also include the act of labeling a composition, i.e., instructing a subject to administer a composition, in a manner

as provided herein for treatment of NASH. In some embodiments, administration can be by parenteral, subcutaneous, intravenous (bolus or infusion), intramuscular, or intraperitoneal routes. Dosage forms for these modes of administration can include conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

[00134] The present invention is also directed to an oral dosage form comprising about 200 mg to about 12 g of DHA. In some embodiments, the oral dosage form is a gel cap. Optionally the gel cap also comprises a colorant, flavoring, and/or antioxidant. In some embodiments, the gel cap is a soft-gelatin capsule made from gelatin, glycerol, and water, and filled with DHA and an antioxidant. In certain embodiments, the gel cap is animal or vegetable derived. In some embodiments, the gel cap comprises a 1 g dosage form, wherein the fill weight of the dosage form is from about 950 mg to about 1050 mg, and wherein the gel cap contains from about 855 mg/g to about 945 mg/g DHA. In some embodiments, the gel cap contains about 900 mg DHA per 1 g of the dosage.

[00135] In some embodiments, the gel cap comprises a capsule preparation, an active, and optionally a colorant and/or antioxidant. In some embodiments, the capsule preparation comprises (i) gelatin (bovine acid hide), glycerin, and purified water; (ii) the active comprises DHA-ethyl ester; (iii) the optional colorant is selected from titanium dioxide, FD&C Yellow #5, FD&C Red 40, and mixtures thereof; and (iv) the antioxidant is ascorbyl palmitate. In some embodiments, the raw materials are USP raw materials.

[00136] In certain embodiments, the gel cap is vegetarian. In some embodiments, the capsule preparation contains no animal products, and comprises glycerol (and/or other polyols), seaweed extract (carrageenan) and water. In some embodiments, the water is purified. In some embodiments, color, flavor and/or sweeteners are added. During encapsulation, in some embodiments, fractionated coconut oil is used as a lubricant.

[00137] In some embodiments, the gelatin capsule comprises a capsule preparation, an active, and optionally a colorant and/or antioxidant. In another embodiment i) the capsule preparation comprises gelatin (bovine acid hide), glycerin, and purified water; ii) the active comprises DHA-ethyl ester; iii) the optional colorant is selected from titanium dioxide, FD&C Yellow #5, FD&C Red 40, and mixtures thereof; and iv) the antioxidant is ascorbyl palmitate. In some embodiments, the raw materials are USP raw materials.

[00138] In some embodiments, the gelatin capsules are soft gelatin capsules of about 1 g, having the specifications within the limits set forth in **Table 7**:

Table 7: Specifications for 1 gram DHA Ethyl Ester Gelatin Capsules

Test	Specification
DHA EE Content, per capsule	855 – 945 mg
Average Fill Weight	950 – 1050 mg
Disintegration	Complies USP
Acid Value	Max 2.0 mg KOH/g
Peroxide Value (PV)	Max 10 meq/kg
Anisidine Value (AV)	Max 20
Microbial Limits Tests	Complies with <61> USP

[00139] Set forth in **Table 8** is a list of components that are, in some embodiments, used in the manufacture of a DHA-ethyl ester soft gelatin capsule, and at least one corresponding function for each component.

Table 8: List of Components in 1 gram DHA Ethyl Ester Soft Gelatin Capsules

Component	Function
900 mg DHA EE	Active
Gelatin, Bovine Acid Hide	Capsule Preparation
Glycerin	Capsule Preparation
Purified Water	Capsule Preparation
Titanium Dioxide	Colorant
FD&C Yellow #5	Colorant
FD&C Red #40	Colorant

[00140] In some embodiments, the gelatin capsule is a soft-gelatin capsule made from gelatin, glycerol, and water, and filled with DHA and an antioxidant. In certain embodiments, the gelatin capsule is animal or vegetable derived. In some embodiments, the gelatin capsule comprises a 0.5 gram dosage form, wherein the fill weight of the weight of the dosage form is from about 450 mg to about 550 mg, and wherein the gelatin capsule comprises from about 430 mg to about 480 mg DHA. In some embodiments, the gelatin capsule comprises about 450 mg DHA per 500 mg of the dosage form. In some embodiments, the gelatin capsule comprises about 450 mg DHA per 500 mg of the dosage form. In some embodiments, the gelatin capsule comprises a 1 gram dosage form, wherein the fill weight of the dosage form is from about 950 mg to about 1050 mg, and

wherein the gelatin capsule comprises from about 860 mg to about 950 mg DHA per 1000 mg of the dosage form. In some embodiments, the gelatin capsule comprises about 900 mg DHA per 1,000 g of the dosage form.

[00141] In certain embodiments, the gelatin capsule is vegetarian. In some embodiments, the capsule preparation comprises no animal products, and comprises glycerol (and/or other polyols), seaweed extract (carrageenan) and water. In some embodiments, the water is purified. In some embodiments, color, flavor and/or sweeteners are added. During encapsulation, in some embodiments, fractionated coconut oil is used as a lubricant.

[00142] In some embodiments, the gelatin capsule comprises a capsule preparation, an active, and optionally a colorant and/or antioxidant. In another embodiment i) the capsule preparation comprises gelatin (bovine acid hide), glycerin, and purified water, ii) the active comprises DHA-EE, iii) the optional colorant is selected from titanium dioxide, FD&C Yellow #5, FD&C Red 40, and mixtures thereof; and iv) the antioxidant is ascorbyl palmitate. In some embodiments, the raw materials are USP raw materials. In some embodiments, the gelatin capsules are soft gelatin capsules of about 1 g, having the specifications within the limits set forth in **Table 8**:

TABLE 8: Specifications for 1 gram DHA Ethyl Ester Gelatin Capsules

TEST	SPECIFICATION
DHA EE CONTENT, PER CAPSULE	855 -945 MG
AVERAGE FILL WEIGHT	950 -1050 MG
DISINTEGRATION	COMPLIES USP
ACID VALUE	MAX 2 MG KOH/G
PEROXIDE VALUE (PV)	MAX 10MEQ/KG
ANISIDINE VALUE (AV)	MAX 20
MICROBIAL LIMITS TESTS	COMPLIES WITH <61> USP

[00143] Set forth in **Table 9** is a list of components that are, in some embodiments, used in the manufacture of a DHA-EE soft gelatin capsule, and at least one corresponding function for each component.

TABLE 9: List of Components in 1 gram DHA Ethyl Ester Soft Gelatin Capsules

COMPONENT	FUNCTION
900 MG DHA EE	ACTIVE
GELATIN, BOVINE ACID HIDE	CAPSULE PREPARATION
GLYCERIN	CAPSULE PREPARATION
PURIFIED WATER	CAPSULE PREPARATION
TITANIUM DIOXIDE	COLORANT
FD&C YELLOW #5	COLORANT COLORANT
FD&C RED #40	

[00144] The present invention is also directed to kits or packages containing one or more dosage forms to be administered according to the methods of the present invention. A kit or package can contain one dosage form, or more than one dosage form (i.e., multiple dosage forms). If multiple dosage forms are present in the kit or package, the multiple dosage forms can be optionally arranged for sequential administration. The kits can contain dosage forms of a sufficient number to provide convenient administration to a subject who has a chronic condition and requires long-term administration of the DHA and NAC of the present invention. For example, in some embodiments, the kit provides dosage forms of a sufficient number for 1, 2, 3 or 4 months of daily administration of the DHA and NAC. In some embodiments of the present invention, the kit comprises dosage forms for shorter periods of administration, e.g., the kit can contain about 7, 14, 21, 28 or more dosage forms for oral administration, each dosage form containing about 200 mg to about 12 g DHA and intended for ingestion on successive days. In some embodiments, the kit comprises 7, 14, 21, 28 or more dosage forms for oral administration, each dosage form containing about 200 mg to about 2 g of NAC.

[00145] The kits of the present invention can optionally contain instructions associated with the dosage forms of the kits. Such instructions can be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical products, which notice reflects approval by the agency of the manufacture, use or sale for human administration to treat a condition or disorder. The instructions can be in any form which conveys information on the use of the dosage forms in the kit according to the methods of

the invention. For example, the instructions can be in the form of printed matter, or in the form of a pre-recorded media device.

[00146] The present invention is also directed to methods of educating consumers about the methods of treating NAFLD and/or NASH of the present invention, the method comprising distributing the DHA dosage forms and NAC with consumer information at a point of sale. In some embodiments, the distribution will occur at a point of sale having a pharmacist or healthcare provider.

[00147] The term "consumer information" can include, but is not limited to, an English language text, non-English language text, visual image, chart, telephone recording, website, and access to a live customer service representative. In some embodiments, consumer information will provide directions for use of the DHA unit dosages and NAC according to the methods of the present invention, appropriate age use, indication, contraindications, appropriate dosing, warnings, telephone number or website address. In some embodiments, the method further comprises providing professional information to relevant persons in a position to answer consumer questions regarding use of the disclosed regimens according to the methods of the present invention. The term "professional information" includes, but is not limited to, information concerning the regimen when administered according to the methods of the present invention that is designed to enable a medical professional to answer customer questions.

EXAMPLES

Example 1

Combination effects of DHA and NAC on high-fat diet induced NASH in rats (28 day treatment)

[00148] Male Sprague Dawley rats are fed either a control diet of standard chow or a high fat (isocaloric diet). The high-fat diet has 59% calories from fat (safflower oil 270 g/kg, soy oil 70 g/kg). The vitamin-A content of the high-fat diet is low to moderate, in order to predispose hepatic stellate cells to a fibrotic state. The vitamin-E content of the high-fat diet is low to moderate since vitamin E is a potent lipid antioxidant. During the study, the rats are housed in small cages to encourage sedentary behavior by restricting physical activity.

- [00149] The dietary treatment spans 28 days. Sample collection is n=10/group. For treatment days 0-7, the control group (n=20) is fed standard chow while the high-fat (HF) group (n=50) is fed the high-fat diet. For days 8-28, there are five dietary treatment groups: (i) control diet (n=10); (ii) HF (n=10); (iii) HF+DHA-ethyl ester (DHA-EE) (n=10); (iv) HF + NAC (n=10); (v) HF + DHA-EE + NAC (n=10). The diets are administered with or without 2 g/kg/day of NAC and with or without 2g/kg/day of DHA-EE. DHA-EE is a concentrated ethyl ester of DHA (900 mg/g) from a microalgal source.
- [00150] At day 7, steatosis is confirmed in the HF group according to body weight, liver cholesterol levels, and liver triglyceride levels. At day 28, the body weight, liver weight, liver triglycerides, serum triglycerides and abdominal fat (or fat pad weight) are measured. In addition, at day 28, the serum levels of alanine transaminase (ALT) are also measured.
- [00151] Liver pathology is measured by hematoxylin-eosin staining (to measure vacuolation). A lipidosis score is determined by the use of Oil Red-O slide staining and portal fibrosis will be measured by Picosirius Red staining.

Example 2

Combination effects of DHA and NAC on rat model of NASH (120 day treatment)

- [00152] Male Sprague Dawley rats are fed either a control diet of standard chow (e.g., 5% energy from fat, 18% energy from protein, and 77% energy from carbohydrates, 3.3 kcal/g) or a high fat diet (58% energy from fat, 18% from protein, 24% from carbohydrates, 5.6 kcal/g). At day 1, the rats weigh approximately 200 g each. For days 0-90, 20 animals will receive the control diet and 50 animals will receive the high fat diet.
- [00153] At 30, 60 and 90 days, animal subjects are tested for alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), total bilirubin (TB) and total cholesterol (TC).
- [00154] For days, 90-120, there will be five dietary treatment groups: (i) control diet (n=10); (ii) HF (n=10); (iii) HF+DHA-EE (n=10); (iv) HF + NAC (n=10); (v) HF + DHA-EE + NAC (n=10). The diets are administered with or without 2 g/kg/day of NAC and with or without 2g/kg/day of DHA-EE.

- [00155] Liver pathology is measured by looking for indications of steatosis, hepatocellular ballooning, lobular inflammation (necroinflammation), fibrosis, and hepatocyte necrosis.
- [00156] It is to be appreciated that the Detailed Description section, and not the Summary and Abstract sections, is intended to be used to interpret the claims. The Summary and Abstract sections can set forth one or more but not all exemplary embodiments of the present invention as contemplated by the inventor(s), and thus, are not intended to limit the present invention and the appended claims in any way.
- [00157] The present invention has been described above with the aid of functional building blocks illustrating the implementation of specified functions and relationships thereof. The boundaries of these functional building blocks have been arbitrarily defined herein for the convenience of the description. Alternate boundaries can be defined so long as the specified functions and relationships thereof are appropriately performed.
- [00158] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.
- [00159] The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.
- [00160] All of the various embodiments or options described herein can be combined in any and all variations. While the invention has been particularly shown and described with reference to some embodiments thereof, it will be understood by those skilled in the art that they have been presented by way of example only, and not limitation, and various changes in form and details can be made therein without departing from the spirit and scope of the invention. Thus, the breadth and scope of the present invention should not

be limited by any of the above described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

[00161] All documents cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued or foreign patents, or any other documents, are each entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited documents.

WHAT IS CLAIMED IS:

1. A method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering
 - (a) a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and
 - (b) an effective amount of N-acetyl L-cysteine (NAC)to the subject in need thereof.
2. The method of claim 1, wherein the DHA is administered in a dosage form.
3. The method of claim 2, wherein the DHA is in the form of an ester.
4. The method of claim 1, wherein the DHA is in the form of a triglyceride.
5. The method of claim 2, wherein the DHA is in the form of free fatty acid.
6. The method of claim 3, wherein the ester is a C₁-C₆ alkyl ester.
7. The method of claim 6, wherein the C₁-C₆ alkyl ester is ethyl ester.
8. The method of any one of claims 2-7, wherein EPA is less than 3% (wt/wt) of the dosage form.
9. The method of any one of claims 2-8, wherein EPA is less than 1% (wt/wt) of the dosage form of DHA.
10. The method of any one of claims 2-9, wherein EPA is less than 0.01% (wt/wt) of the dosage form of DHA.

11. The method of any one of claims 2-10, wherein EPA is not detectable in the dosage form of DHA.
12. The method of claim any one of claims 2-11, wherein the dosage form of DHA is administered substantially free of arachidonic acid (ARA).
13. The method of any one of claims 2-11, wherein ARA is less than about 2% (wt/wt) of the dosage form of DHA.
14. The method any one of claims 1-13, wherein the DHA is derived from an algal source.
15. The method of claim 14, wherein the algal source is *Crypthecodinium cohnii*, *Thraustochytrium*, or *Schizochytrium sp.*
16. The method of any one of claims 1-13, wherein the DHA is derived from a plant source.
17. The method of claim 16, wherein the plant source is a soybean.
18. The method of any one of claims 2-17, wherein about 200 mg to about 1 g of DHA is administered per day to the subject.
19. The method of claim 18, wherein the dosage form of DHA has a total weight of about 200 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 550 mg, about 600 mg, about 650 mg, about 700 mg, about 750 mg, about 800 mg, about 850 mg, about 900 mg, about 950 mg, about 1.0 g , or about 1.05 g.
20. The method of any one of claims 2-17, wherein about 840 mg to about 4 g of DHA is administered per day to the subject.

21. The method of claim 20, wherein about 840 mg to about 1.5 g of DHA is administered per day to the subject.
22. The method of claim 20, wherein about 840 mg to about 1.0 g of DHA is administered per day to the subject.
23. The method of claim 1, wherein the DHA in the dosage form is about 30% to about 99.5% (wt/wt) of the total fatty acid content of the dosage form.
24. The method of claim 23, wherein the DHA in the dosage form is about 35% to about 65% (wt/wt) of the total fatty acid content of the dosage form.
25. The method of any one of claims 23-24, wherein the dosage form is characterized by one or more of the following amounts of fatty acids or esters thereof:
 - (a) capric acid is about 1% (wt/wt) or less;
 - (b) lauric acid is about 1% (wt/wt) or less;
 - (c) myristic acid is about 1% (wt/wt) or less;
 - (d) palmitic acid is about 1% (wt/wt) or less;
 - (e) palmitoleic acid is about 1% (wt/wt) or less;
 - (f) stearic acid is about 1% (wt/wt) or less;
 - (g) oleic acid is about 1% (wt/wt) or less;
 - (h) linoleic acid is about 1% (wt/wt) or less;
 - (i) α -linolenic acid is about 1% (wt/wt) or less;
 - (j) docosapentaenoic acid 22:5n-3 (DPAn3) is about 1% (wt/wt) or less;
 - (k) docosapentaenoic acid 22:5n-6 (DPAn6) is about 1% (wt/wt) or less; and
 - (l) 4,7,10,13,16,19,22,25 octacosaoctaenoic acid (C28:8) is less than about 1% (wt/wt)of the total fatty acid content of the dosage form.

26. The method of claim 1, wherein the DHA in the dosage form comprises about 40% to about 50% (wt/wt) of the total weight of the dosage form.
27. The method of claim 26, wherein the DHA in the dosage form comprises about 40% to about 45% (wt/wt) of the total fatty acid content of the dosage form.
28. The method of any one of claims 26-27, wherein the dosage form is characterized by one or more of the following amounts of fatty acid or esters thereof:
- (a) capric acid (C10:0) is about 2% (wt/wt) or less;
 - (b) lauric acid (C12:0) is about 6% (wt/wt) or less;
 - (c) myristic acid (C14:0) is about 20% (wt/wt) or less;
 - (d) palmitic acid (C16:0) is about 20% (wt/wt) or less;
 - (e) palmitoleic acid (C16:1n-7) is about 3% (wt/wt) or less;
 - (f) stearic acid (C18:0) is about 2% (wt/wt) or less;
 - (g) oleic acid (C18:1n-9) is about 40% (wt/wt) or less;
 - (h) linoleic acid (C18:2) is about 5% (wt/wt) or less;
 - (i) arachidonic acid (C20:4) is about 0.1% (wt/wt) or less;
 - (j) eicosapentaenoic acid (C20:5) is about 3% (wt/wt) or less;
 - (k) decosapentaenoic acid (22:5n-6) is about 0.1% (wt/wt) or less;
 - (l) nervonic acid (C24:1) is about 2% (wt/wt) or less; and
 - (m) other fatty acids are about 3% (wt/wt) or less
- of the total fatty acid content of the dosage form.
29. The method of claim 1, wherein the DHA in the dosage form comprises about 35% to about 45% (wt/wt) of the total weight of the dosage form.
30. The method of claim 29, wherein the DHA in the dosage form comprises about 35% to about 45% (wt/wt) of the total fatty acid content of the dosage form.

31. The method of any one of claims 29-30, wherein the dosage form is characterized by one or more of the following amounts of fatty acid or esters thereof:
- (a) capric acid (C10:0) is 0.01% (wt/wt) or less;
 - (b) lauric acid (C12:0) is about 0.5% (wt/wt) or less;
 - (c) myristic acid (C14:0) is about 12% (wt/wt) or less;
 - (d) palmitic acid (C16:0) is about 28% (wt/wt) or less;
 - (e) palmitoleic acid (C16:1n-7) is about 0.5% (wt/wt) or less;
 - (f) stearic acid (C18:0) is about 2% (wt/wt) or less;
 - (g) oleic acid (C18:1n-9) is about 8% (wt/wt) or less;
 - (h) linoleic acid (C18:2) is about 2% (wt/wt) or less;
 - (i) arachidonic acid (C20:4) is about 2% (wt/wt) or less;
 - (j) eicosapentaenoic acid (C20:5) is about 3% (wt/wt) or less;
 - (k) decosapentaenoic acid (22:5n-6) is about 18% (wt/wt) or less;
 - (l) nervonic acid (C24:1) is about 0.01% (wt/wt) or less; and
 - (m) other fatty acids are about 10% (wt/wt) or less
- of the total fatty acid content of the dosage form.
32. The method of claim 1, wherein the DHA in the dosage form comprises about 55% (wt/wt) of the total weight of the dosage form.
33. The method of claim 1, wherein the DHA in the dosage form comprises about 55% to about 67% (wt/wt) of the total fatty acid content of the dosage form.
34. The method of any one of claims 32-33, wherein the dosage form is characterized by one or more of the following amounts of fatty acid or esters thereof:
- (a) capric acid (C10:0) is about 2% (wt/wt) or less;
 - (b) lauric acid (C12:0) is about 6% (wt/wt) or less;

- (c) myristic acid (C14:0) is about 20% (wt/wt) or less;
 - (d) palmitic acid (C16:0) is about 15% (wt/wt) or less;
 - (e) palmitoleic acid (C16:1n-7) is about 5% (wt/wt) or less;
 - (f) stearic acid (C18:0) is about 2% (wt/wt) or less;
 - (g) oleic acid (C18:1n-9) is about 20% (wt/wt) or less;
 - (h) linoleic acid (C18:2) is about 2% (wt/wt) or less;
 - (i) arachidonic acid (C20:4) is about 0.1% (wt/wt) or less;
 - (j) eicosapentaenoic acid (C20:5) is about 0.1% (wt/wt) or less;
 - (k) decosapentaenoic acid (22:5n-6) is about 0.1% (wt/wt) or less; and
 - (l) other fatty acids are about 3% (wt/wt) or less
- of the total fatty acid content of the dosage form.
35. The method of claim 1, wherein the DHA in the dosage form comprises a DHA/DPA_n6 ratio (wt/wt) of about 2.5 to about 2.7.
36. The method of claim 35, wherein the DHA in the dosage form comprises greater than about 67% to about 72% (wt/wt) of the total fatty acid content of the dosage form.
37. The method of any one of claims 35-36, wherein the dosage form comprises DPA_n6 of about 15% to about 30% (wt/wt) of the total fatty acid content of the dosage form.
38. The method of claim 21, wherein the DHA in the dosage form comprises greater than about 85% (wt/wt) of the total weight of the dosage form.
39. The method of claim 21, wherein the DHA in the dosage form comprises about 85% to about 96% (wt/wt) of the total weight of the dosage form.
40. The method of claim 38, wherein the DHA in the dosage form comprises about 85% to about 99.5% (wt/wt) of the total fatty acid content of the dosage form.

41. The method of any one of claims 38-39, wherein the dosage form is characterized by one or more of the following amounts of fatty acid or esters thereof:
- (a) capric acid (C10:0) is about 0.1% (wt/wt) or less;
 - (b) lauric acid (C12:0) is about 0.1% (wt/wt) or less;
 - (c) myristic acid (C14:0) is about 0.1% (wt/wt) or less;
 - (d) palmitic acid (C16:0) is about 0.5% (wt/wt) or less;
 - (e) palmitoleic acid (C16:1n-7) is about 0.5% (wt/wt) or less;
 - (f) stearic acid (C18:0) is about 0.5% (wt/wt) or less;
 - (g) oleic acid (C18:1n-9) is about 4% (wt/wt) or less;
 - (h) linoleic acid (C18:2) is about 0.1% (wt/wt) or less;
 - (i) arachidonic acid (C20:4) is about 0.1% (wt/wt) or less;
 - (j) eicosapentaenoic acid (C20:5) is about 0.1% (wt/wt) or less;
 - (k) decosapentaenoic acid (22:5n-6) is (wt/wt) or less; and
 - (l) other fatty acids are about 1% (wt/wt) or less
- of the total fatty acid content of the dosage form.
42. The method of any one of claims 38-40, wherein the DHA comprises a DHA ethyl ester.
43. The method of claim 41, wherein the dosage form or unit dose comprises about 430 mg to about 480 mg of DHA ethyl ester.
44. The method of claim 41, wherein the dosage form or unit dose comprises about 860 mg to about 950 mg of the DHA ethyl ester.
45. The method of claim 43, wherein the dosage form or unit dose comprises about 870 mg to about 930 mg of the DHA ethyl ester.

46. The method of any one of claims 38-44, wherein the dosage form or unit dose further comprises one or more tocopherols, one or more tocotrienols, or a combination thereof.
47. The method of any one of claims 1-45, wherein the DHA and the effective amount of NAC are simultaneously co-administered.
48. The method of any one of claims 1-46 wherein the dosage form is administered once per day.
49. The method of claim 47, wherein the dosage form is administered daily for the remainder of the subject's lifetime.
50. The method of claim 47, wherein the dosage form is administered daily for 1 to 10 years.
51. The method of claim 47, wherein the dosage form is administered daily for 1 to 12 consecutive months.
52. The method of claim 47, wherein the dosage form is administered daily for at least 6 consecutive months.
53. The method of claim any one of claims 1-51, wherein the dosage form is an oral dosage form.
54. The method of claim 52, wherein the oral dosage form is a gelatin capsule, or caplet.
55. The method of any one of claims 1-53, wherein the subject has an alanine transaminase (ALT) plasma level of greater than about 40 IU/L.

56. The method of any one of claims 1-54, wherein the subject has been diagnosed with non-alcoholic steatohepatitis (NASH).
57. The method of any one of claims 1-55, wherein the administration of DHA reduces the incidence and severity of hepatocellular rarefaction and hepatocellular vacuolation in the subject.
58. A method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering
- (a) a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and
 - (b) about 200 mg to about 2 g of N-acetyl L-cysteine (NAC)
- to the subject in need thereof.
59. A method of enhancing liver health and/or liver function in a subject in need thereof, the method comprising administering
- (a) a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and
 - (b) an effective amount of N-acetyl L-cysteine (NAC)
- to the subject in need thereof.
60. A method of enhancing liver health and/or liver function in a subject in need thereof, the method comprising administering
- (a) a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA), and
 - (b) about 200 mg to about 2 g of N-acetyl L-cysteine (NAC)
- to the subject in need thereof.

61. A composition comprising
- (a) about 200 mg to about 700 mg docosahexaenoic acid (DHA), and
 - (b) about 200 mg to about 700 mg of N-acetyl L-cysteine (NAC).
62. The composition of claim 60, wherein the composition comprises less than about 3% (wt/wt) eicosapentaenoic acid (EPA) of the total fatty acid content.
63. The composition of any one of claims 60-61, wherein the composition comprises less than about 2% (wt/wt) arachidonic acid (ARA) of the total fatty acid content.
64. The composition of any one of claims 60-62, wherein the composition comprises less than about 2% (wt/wt) docosapentaenoic acid 22:5n-6 (DPAn6) of the total fatty acid content.
65. The composition of any one of claims 60-63, wherein the DHA is derived from an algal source.
66. The composition of any one of claims 60-63, wherein the DHA is derived from a plant source.
67. The composition of any one of claims 60-65, wherein the DHA is in the form of an ethyl ester.
68. The composition of any one of claims 60-66, wherein the DHA is 90% (wt/wt) or greater of the total fatty acid content.
69. A method for treating non-alcoholic fatty liver disease (NAFLD) in a subject in need thereof, the method comprising administering

- (a) a dosage form of about 200 mg to about 12 g of docosahexaenoic acid (DHA) substantially free of eicosapentaenoic acid (EPA) to the subject in need thereof.
70. The method of claim 69, wherein the DHA in the dosage form comprises greater than about 85% (wt/wt) of the total weight of the dosage form.
71. The method of claim 70 wherein the dosage form is administered as a unit dose comprising about 840 mg to about 1.5 g of DHA and is administered at least once per day to the subject.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/23434

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 37/00, A61K 31/20 (2011.01)

USPC - 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 -- 514/560

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC -- 435/4, 435/125, 435/113, 514/558 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files -- 654, 652, 349, 35, 65, 155; USPTO Web Page; Google Scholar;
Search terms -- DHA, EPA, DPA, omega-3, omega-6, ethyl ester, triglyceride, free fatty acid, non-alcoholic fatty liver, steatohepatitis, N-acetylcysteine, dosage, saturated fatty acid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2009/0182022 A1 (RONGEN et al.) 16 July 2009 (16.07.2009) para [0002]-[0003], [0015], [0017], [0019], [0022], [0026], [0028]-[0030], [0032], [0034]	69-71 ----- 1-8, 23-37, 58-64
Y	US 6,646,013 B1 (BARKER et al.) 11 November 2003 (11.11.2003) col 12, ln 25-45 (Table 1); col 17, ln 5-20	1-8, 23-37, 58-64
Y	US 2009/0023808 A1 (RAMAN et al.) 22 January 2009 (22.01.2009) para [0077], [0131], [0138], [0141], [0143], [0150]	25, 28, 31, 34-37

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

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search
10 March 2011 (10.03.2011)

Date of mailing of the international search report

30 MAR 2011

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/23434

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 9-22, 38-57 and 65-68
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.