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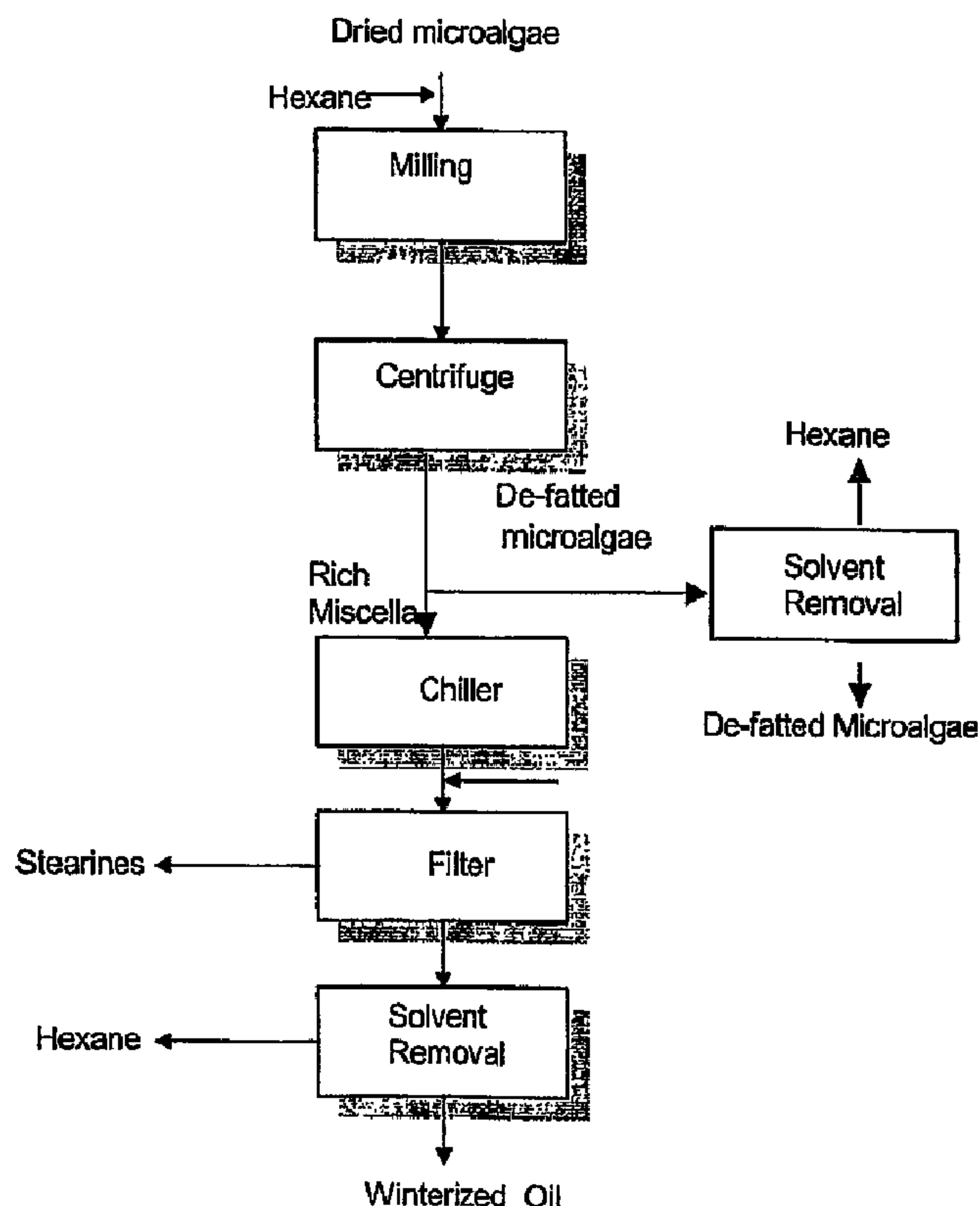
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(54) Title: EXTRACTION AND WINTERIZATION OF LIPIDS FROM OILSEED AND MICROBIAL SOURCES



Overview of the DHA-rich lipid extraction process using hexane

(57) Abrégé/Abstract:

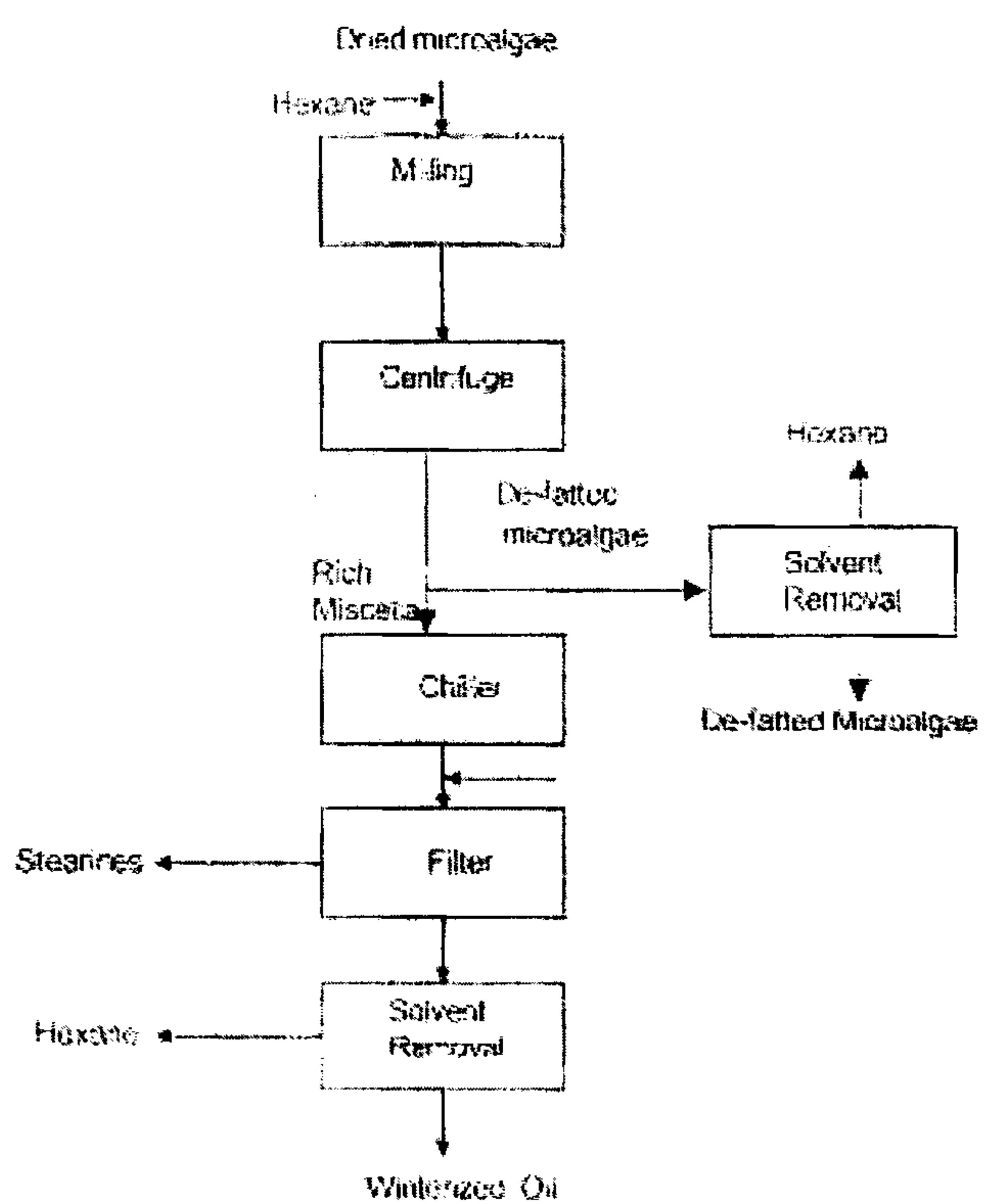
A process for purifying a lipid composition having predominantly neutral lipid components having at least one long chain polyunsaturated fatty acid is disclosed. The process employs contacting the lipid composition with polar solvent, such as acetone,

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wherein the solvent is selected such that contaminants are less soluble in the solvent than in the long chain polyunsaturated fatty acid. The process is typically conducted at cooler temperatures, including about 0°C. Upon precipitation of the contaminants from the lipid composition, a separation is conducted to remove the precipitated material from the lipid composition. The long chain polyunsaturated fatty acids can include ARA, DPA, EPA and/or DHA. The process effectively winterizes lipid compositions, thereby reducing the tendency of such compositions to become hazy.

ABSTRACT

A process for purifying a lipid composition having predominantly neutral lipid components having at least one long chain polyunsaturated fatty acid is disclosed. The process employs contacting the lipid composition with polar solvent, such as acetone, wherein the solvent is selected such that contaminants are less soluble in the solvent than in the long chain polyunsaturated fatty acid. The process is typically conducted at cooler temperatures, including about 0°C. Upon precipitation of the contaminants from the lipid composition, a separation is conducted to remove the precipitated material from the lipid composition. The long chain polyunsaturated fatty acids can include ARA, DPA, EPA and/or DHA. The process effectively winterizes lipid compositions, thereby reducing the tendency of such compositions to become hazy.



Extraction and Winterization of Lipids From Oilseed and Microbial Sources

FIELD OF THE INVENTION

The present invention is directed to the extraction and purification of lipids, and in particular, lipids containing long chain polyunsaturated fatty acids (LCPUFAs). In particular, processes are provided for obtaining high concentrations of desired LCPUFAs and low concentrations of undesired compounds such as trisaturated glycerides.

BACKGROUND OF THE INVENTION

In general, winterization is the name given to the process of removing sediment that appears in vegetable oils at low temperature. It originated from the early practice of allowing cottonseed oil to remain in outdoor storage during the cool winter months and filtering off the sediment-free oil. Dry fractional crystallization is a process wherein triglycerides with the highest melting temperature preferentially crystallize during cooling from a neat liquid (e.g., liquid lipid). After crystallization is complete, the solid phase is separated from the liquid phase by one of several types of physical processes. Alternatively, solvent crystallization is used to promote triglyceride crystal formation, because triglycerides at low temperature generally form more stable crystals with solvent than without solvent.

Docosahexaenoic acid (DHA)-rich lipid was extracted using conventional techniques and solvents (e.g., hexane) from *Schizochytrium* sp. biomass produced by fermentation, and the resulting extracted lipid was winterized by chilling it to -2 to 2°C followed by centrifugation. The lipid was then refined, bleached and deodorized, and put into gelatin capsules for sale as nutritional supplements. A problem arose with this product in that a haze would form in the product over time.

In one process for recovering lipids from biomass, as illustrated in Figure 1, dried microalgae are suspended in commercial-grade n-hexane and wet milled. Hexane primarily extracts triglycerides, diglycerides, monoglycerides and esterified sterols, although other components of the total lipid fraction, such as phospholipids, free sterols and carotenoids, can also be extracted to a lesser degree. Centrifugation is employed to separate spent biomass from a lipid-rich miscella. The resultant mixture of lipid and solvent is referred to as miscella. The lipid content of the clarified miscella is adjusted to about 45 wt% using n-hexane. The miscella is winterized, in particular, the miscella is chilled to approximately -1°C, and held for 8 to 12 hours, to crystallize any saturated fats,

or high melting point components. The miscella is then filtered to remove the crystallized stearine phase. Hexane is removed from the miscella, leaving behind the winterized lipid.

As illustrated in Figure 2, the winterized lipid is heated and treated with citric acid or phosphoric acid to hydrate any phosphatides present in the lipid. Sodium hydroxide is 5 added to neutralize any free fatty acids present. The resulting gums (hydrated phosphatides) and soapstock (neutralized fatty acids) are removed using a centrifuge. The lipid is mixed with water and re-centrifuged to remove any residual gum/soapstock. This step can be carried out with the first centrifugation. The refined lipid is bleached 10 with silica and bleaching clay following pre-treatment with citric acid, to remove peroxides, color compounds, and traces of soapstock, phospholipids and metals. Filter aid is added at the end of the cycle to facilitate removal of the spent bleaching compounds from the lipid *via* filtration.

An additional step can be performed, where the bleached lipid is chilled to from about 5°C to about 15°C and held for about 6 to about 8 hours to crystallize any 15 remaining stearines or waxes, if it is apparent that a sediment layer will form upon standing. Filter aid can be used to facilitate removal of the crystals *via* filtration, if this step is performed.

A deodorizer, operated at elevated temperatures under high vacuum, is used to 20 destroy peroxides, which if left intact could later decompose and initiate free radical reactions. This step also removes any remaining low molecular weight compounds that can cause off-odors and flavors. Contact times in the deodorizer are minimized to prevent the formation of trans-fatty acids. Safe and suitable food approved antioxidants are added. The stabilized lipid is packaged in a phenolic-lined metal container under a nitrogen atmosphere to prevent oxidation.

25 The haze that formed in the lipid-filled gelatin capsules was analyzed and found to be composed of crystals of triglycerides containing myristic (14:0) and palmitic (16:0) fatty acids, a trisaturated fatty acid glyceride. These crystals had a melting point of about 50-55°C. The trisaturated glycerides comprised 6-8% of the crude extracted lipid. The above-described winterization process lowered the concentration of these trisaturated 30 glycerides to <1%; however, not low enough to completely eliminate haze formation in the lipid. Additionally, about 30% of the lipids, and a corresponding 30% of the DHA, is removed in this traditional hexane (55% hexane and 45% crude oil) winterization process. Another problem was that when the temperature was lowered to crystallize the remaining <1% of the trisaturated triglycerides, more of the desired LCPUFA, e.g., disaturated

triglycerides containing one DHA molecule, would also crystallize out. This would cause significant losses of the target product, DHA. Losses could be an additional 8-10% of the lipids,. So by trying to solve one problem, another was created. It would be desirable to have a process by which the LCPUFA level could be maintained at a desirably high level and the haze could be reduced or eliminated.

SUMMARY

An object of the present invention is to provide extraction and winterization of lipids from oilseed and microbial sources.

The present invention includes a process for purifying a lipid composition having predominantly neutral lipid components wherein the composition contains at least one long chain polyunsaturated fatty acid (LCPUFA) and at least one other compound. The process includes contacting the lipid composition with a polar solvent and the solvent is selected such that the other compound is less soluble in the solvent than is the LCPUFA. For example, the polar solvent can be selected from acetone, isopropyl alcohol, methanol, ethanol, ethyl acetate and mixtures thereof. The process further includes maintaining the lipid composition at a temperature range effective to precipitate at least a portion of the other compound. For example, the temperature range can be from about -20°C to about 50°C, from about -5°C to about 20°C, from about -5°C to about 5°C or about 0°C. The process then includes removing at least a portion of the other compound from the lipid composition to form a lipid product. The process can be specifically for the reduction of the formation of haze in a lipid composition in which the compound being removed is a haze-forming compound.

In accordance with another aspect of the invention, there is provided a process for reducing the formation of haze in a lipid composition, wherein said lipid composition contains at least one long chain polyunsaturated fatty acid (LCPUFA) and at least one haze-forming compound, said process comprising the steps:

- (a) contacting said lipid composition with a polar solvent, wherein said haze-forming compound is less soluble than said LCPUFA in said polar solvent;
- (b) maintaining said lipid composition at a temperature range effective to precipitate at least a portion of said haze-forming compound; and
- (c) removing at least a portion of said haze-forming compound from said lipid composition to form a lipid product.

In various embodiments, the lipid composition can include at least 50% or 85% neutral lipid, or at least 50% triglyceride. The concentration of LCPUFA, on a weight percentage basis, can be greater after the process than before, and the concentration of the other compound, on a weight percentage basis, can be less after the process than before. For example, the total concentration of any phosphorus-containing compounds present in the lipid, on a weight percentage basis, is less after the process than before. The process of the present invention can result in an acceptable product with less downstream processing required, such as with reduced degumming or no degumming required.

The LCPUFA can be arachidonic acid (ARA), omega-6 docosapentaenoic acid (DPA(n-6)), omega-3 docosapentaenoic acid (DPA(n-3)), eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA). The other compound can be trisaturated glycerides, phosphorus-containing materials, wax esters, saturated fatty acid containing sterol esters, sterols, squalene, and/or hydrocarbons. Alternatively, the other compound can be

trisaturated glycerides, phosphatides and wax esters. Alternatively, the other compound can be trisaturated glycerides of lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) fatty acids and/or mixtures thereof. In a particular embodiment, the lipid composition initially comprises at least one LCPUFA and at least one trisaturated glyceride. The LCPUFA can be obtained from a LCPUFA-containing biomaterial selected from LCPUFA-containing microbial biomass and oilseeds from plants that have been genetically modified to produce LCPUFA-containing lipid. Also, the LCPUFA can be obtained from plants that have been modified with LCPUFA-producing genes from microbes. In another embodiment, the LCPUFA can be obtained from a source selected from the group consisting of thraustochytrid biomass, dinoflagellate biomass, *Mortierella* biomass, and oilseeds from genetically modified plants containing genes from thraustochytrids, dinoflagellates or *Mortierella*. In a further embodiment, the LCPUFA is obtained from the group comprising *Schizochytrium*, *Thraustochytrium* or *Cryptocodinium cohnii* biomass or oilseeds from genetically modified plants containing genes from *Schizochytrium* or *Thraustochytrium*.

In various embodiments of the invention, the solvent:lipid composition ratio is from about 1:10 to about 20:1, from about 1:8 to about 10:1, from about 1:5 to about 5:1, from about 1:2 to about 2.5:1, or about 1:1. In other embodiments, the time of contact between the solvent and the lipid composition is from about 0.5 to about 12 hours, from about 2 to about 6 hours, or about 4 hours.

In another embodiment of the invention, lipid is extracted using the polar solvent at low temperatures such that triglyceride molecules containing the LCPUFA are selectively extracted and other compounds that are not soluble in the polar solvent are not extracted. In a further embodiment, the lipid composition is extracted from a biomass and cellular debris and precipitated other compounds are separated from a miscella comprising the LCPUFA and the polar solvent.

A further embodiment of the invention includes employing the polar solvent to recover lipid in an extraction process conducted at temperatures that solubilize substantially all triglyceride components; forming a miscella comprising a mixture of the lipid composition and the polar solvent; cooling the miscella to selectively precipitate the undesired compounds; and separating the precipitated other compounds from the miscella. In this embodiment, the lipid composition can be extracted from biomass and cellular debris and precipitated other compounds are separated from a miscella comprising the LCPUFA and the polar solvent.

Another embodiment of the invention includes employing the polar solvent to recover lipid from a biomass in an extraction process conducted at temperatures that solubilize substantially all triglyceride components, forming a miscella comprising a mixture of the lipid composition, the polar solvent and cellular debris. The process 5 further includes separating the cellular debris from the miscella and cooling the miscella to selectively precipitate the undesired compounds. Finally, the precipitated other compounds are separated from the miscella.

A further embodiment of the invention includes employing a nonpolar solvent to recover lipid in an extraction process conducted at temperatures that solubilize 10 substantially all triglyceride components, forming a miscella comprising a mixture of the lipid composition and the nonpolar solvent. The process further includes removing most of the nonpolar solvent from the miscella, adding a polar solvent to the miscella, and cooling the miscella to selectively precipitate the undesired compounds. Finally, the precipitated other compounds are separated from the miscella. A still further embodiment 15 of the invention includes employing a nonpolar solvent to recover lipid in an extraction process conducted at temperatures that solubilize substantially all triglyceride components, forming a miscella comprising a mixture of the lipid composition and the nonpolar solvent and winterizing the miscella. Most of the nonpolar solvent is removed from the miscella, and a polar solvent is added to it. The miscella is cooled to selectively 20 precipitate the undesired compounds which are separated from the miscella. When the nonpolar solvent is removed from the miscella, the residual nonpolar solvent after removal is from about 0 to about 4 weight percent or from about 1 to about 4 weight percent.

In the various embodiments of the invention using a nonpolar solvent, the 25 nonpolar solvent can be hexane. In various embodiments of the invention employing a separating or removing step for the precipitated other compound, the step can be a liquid/solid separation technique, such as centrifugation, filtering or combinations thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1 is a flow diagram of a prior extraction process.

Figure 2 is a flow diagram of a prior refining, bleaching and deodorizing process.

Figure 3 is a flow diagram of a DHA-rich lipid extraction process of the present invention using acetone in one step.

Figure 4 is a flow diagram of a DHA-rich lipid extraction process of the present invention using acetone in two steps.

Figure 5 is a flow diagram of a DHA-rich lipid hexane extraction process and acetone winterization process of the present invention.

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DESCRIPTION OF THE INVENTION

In accordance with the present invention, processes are provided for preferentially reducing the level of undesired components in a lipid, while maintaining high levels of desired LCPUFAs. As used herein, LCPUFAs are fatty acids with 20 or more carbon atoms and two (preferably three) or more double bonds. The LCPUFAs can be in a variety of forms, such as phospholipids, free fatty acids and esters of fatty acids, including triglycerides of fatty acids. It will be appreciated that when referring to the desired LCPUFA, what is meant is the LCPUFA in the form that exists in the lipid, most typically a triglyceride, and to a lesser extent mono- and diglycerides. Preferably, the concentration of the desired LCPUFA, as measured on a weight percent basis, is higher in the resulting lipid product than it is in the starting lipid composition. The undesired components are preferably trisaturated glycerides, such as trisaturated glycerides of lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) fatty acids and mixtures thereof. Examples of other undesired components, in addition to trisaturated glycerides, include phosphorus-containing compounds (e.g., phosphatides or phospholipids), wax esters, saturated fatty acid containing sterol esters, sterols, squalene, hydrocarbons and the like. Preferably, two or more of the undesired compounds are reduced in the resulting product as compared to the starting lipid, as measured on a weight percent basis. As used herein, amounts will generally be on a weight percent basis, unless indicated otherwise.

25 In a preferred embodiment of the present invention the resulting product is subject to less haze or cloudiness when compared to the starting lipid. As a result of the process of the present invention, subsequent processing steps such as refining, can be reduced or eliminated. For example, subsequent processing steps such as bleaching and/or deodorizing can help reduce or eliminate the refining (or degumming) step. An example 30 of the refining, bleaching and deodorizing process is set forth in comparative Example 2. If the refining process is not eliminated, it can be reduced by reducing the amount of caustic employed. While not wishing to bound by any theory, it is believed that a primary cause of haze or cloudiness results from trisaturated triglycerides. It does not appear to be as important to reduce the mono- and di- substituted triglycerides.

As used herein the term "lipids" will refer generally to a variety of lipids, such as phospholipids; free fatty acids; esters of fatty acids, including triglycerides of fatty acids; sterols; pigments (e.g., carotenoids and oxycarotenoids) and other lipids, and lipid associated compounds such as phytosterols, ergothionine, lipoic acid and antioxidants 5 including beta-carotene, tocotrienols, and tocopherol. Preferred lipids and lipid associated compounds include, but are not limited to, cholesterol, phytosterols, desmosterol, tocotrienols, tocopherols, ubiquinones, carotenoids and xanthophylls such as beta-carotene, lutein, lycopene, astaxanthin, zeaxanthin, canthaxanthin, and fatty acids 10 such as conjugated linoleic acids, and omega-3 and omega-6 highly unsaturated fatty acids such as eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid, arachidonic acid, stearidonic acid, dihomogammalinolenic acid and gamma-linolenic acid or mixtures thereof. For the sake of brevity, unless otherwise stated, the term "lipid" 15 refers to lipid and/or lipid-associated compounds.

The undesirable components share the common characteristic of being relatively 15 insoluble in cold acetone or in an analogous polar solvent. On the other hand, desired LCPUFAs, such as arachidonic acid (ARA), omega-6 docosapentaenoic acid (DPA(n-6)), omega-3 docosapentaenoic acid (DPA(n-3)), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), are soluble in cold acetone or in an analogous solvent. The 20 key characteristic of the solvent, whether it is acetone or an analogous polar solvent, is that the desirable LCPUFAs are soluble in the solvent at the desired temperatures, and the undesirable compounds are not soluble in the solvent at the same temperatures. A useful guide is to select solvents that have dielectric constants close to those of acetone or ethyl acetate. Preferred solvents for use in connection with the present invention include 25 acetone and analogous polar solvents such as isopropyl alcohol, methanol, ethanol, ethyl acetate or mixtures of these solvents. The solvents are all polar, and the LCPUFAs, with their double bonds and long carbon chains, are also polar and therefore soluble in the polar solvents. However, if the solvents are too polar, the LCPUFAs may not dissolve. The solvent is also preferably useful in food applications.

It was unexpectedly found that acetone can be used to selectively precipitate the 30 trisaturated glycerides from the crude lipid. When an unwinterized lot of DHA-rich lipid from *Schizochytrium* sp. was treated with 5 volumes of acetone and chilled, essentially all of the trisaturated glycerides were removed by crystallization followed by centrifugation. This process removed little or none of the DHA-containing triglycerides. The resulting

winterized lipid contained 41% DHA as compared to 37% by the standard winterization process.

There are ways to further utilize this discovery by combining acetone or analogous solvent extraction with "in-situ" winterization concepts to better improve the 5 recovery efficiency of long chain polyunsaturated fatty acid containing triglycerides at the expense of trisaturated glycerides or from triglycerides containing two saturated fatty acids and one mono-unsaturated fatty acid. One advantage of the process of the present invention is that less of the desired LCPUFAs are lost. For example, in prior processes about 30% of the extracted lipid, which contained the desired LCPUFAs, was lost during 10 winterization. In contrast, the embodiment of the process of the present invention (i.e., hexane extraction followed by acetone winterization) that is most directly comparable to the prior process results in the loss of only about 7% to about 10% of the starting extracted lipid as a result of the acetone winterization. As a result, in this embodiment of the present invention, about 40% or more reduction in yield loss is realized. This is a 15 significant improvement over the prior process (hexane extraction and winterization plus full refining, bleaching and deodorizing (RBD)). The largest loss of both DHA and lipid is incurred in the winterization step of the prior process.

First, in a preferred process, lipid is extracted using acetone or analogous polar solvent (instead of hexane) at low temperatures such that triglyceride molecules 20 containing LCPUFA are selectively extracted from *Schizochytrium* sp. biomass. A flow diagram of such a process is illustrated in Figure 3. Due to the selectivity of acetone at low temperature (trisaturated glycerides are not soluble in cold acetone, while LCPUFA-containing triglycerides are soluble in cold acetone), it is feasible to selectively remove the LCPUFA-containing triglyceride from biomass and thus eliminate the need for a 25 separate winterization step. The solvent extraction can be conducted in any suitable manner. For example, the dry biomass can be subjected to mechanical (e.g., in a mill or homogenizer) or chemical (e.g., using an acid, enzyme or base) lysing in the presence of a cold solvent. The cellular debris and precipitated trisaturated glycerides are separated from the miscella in one step. Post processing steps, such as purification by refining, 30 bleaching and deodorizing, can be performed, if desired.

A second option is to utilize acetone or analogous polar solvent to quantitatively recover lipid from biomass in a conventional extraction process (including any type of solvent grinding technique). This extraction is conducted at temperatures that solubilize all triglyceride components. Prior to removing cellular debris from the miscella (lipid

containing triglycerides in solvent), the miscella is chilled to selectively remove the trisaturated glycerides. The chilled miscella is then centrifuged, filtered, or separated using other techniques to remove both the cellular debris and trisaturated glyceride component. This option combines the concept of extraction and winterization into one 5 step.

A third option is to utilize acetone or analogous polar solvent to quantitatively recover lipid from biomass in a conventional extraction process (including any type of solvent grinding technique). This extraction is conducted at temperatures that solubilize all triglyceride components. The cellular debris from the miscella (lipid containing 10 triglycerides in solvent) is removed using conventional separation techniques. The miscella is then chilled to crystallize the trisaturated glycerides, which are removed by centrifugation, filtration, or separation using other techniques. This option utilizes extraction and winterization in two stages; however, acetone or an analogous polar solvent is utilized to accomplish both tasks. A flow diagram illustrating such a process is 15 shown in Figure 4.

A fourth option is to utilize a nonpolar solvent such as hexane (e.g., n-hexane, isohexane or a combination thereof) as an extraction solvent and utilize acetone as a winterization solvent. Preferably, at least 95%, more preferably at least 96%, more 20 preferably at least 97%, more preferably at least 98% and more preferably at least 99% of the nonpolar solvent is removed prior to winterization. The winterization step can be employed at any stage prior to deodorization. A flow diagram illustrating such a process is shown in Figure 5.

A fifth option is to utilize conventional hexane extraction and hexane-based winterization to remove the majority of the trisaturated glyceride component and employ 25 a “polishing” step prior to deodorization to remove the small amounts of trisaturated glycerides contributing to the haze formation in the lipid. The polishing step employs acetone and/or an analogous solvent. This option removes the problems caused by haze, but the lipid level is also reduced.

Preferably, the lipid composition initially comprises at least one LCPUFA and at 30 least one trisaturated glyceride. Preferably, the other or undesired compound results in the formation of haze when present in the initial concentration in the initial lipid composition. Preferably, the LCPUFA-containing biomaterial for lipid extraction is selected from the group including: LCPUFA-containing microbial biomass or oilseeds from plants that have been genetically modified to produce LCPUFA containing lipids,

particularly plants that have been modified with the LCPUFA-producing genes from microbes (algae, fungi, protists, or bacteria). More preferably, the LCPUFA-containing biomaterial for lipid extraction is selected from the group including thraustochytrid biomass, dinoflagellate biomass and/or *Mortierella* biomass, and/or oilseeds from genetically modified plants containing genes from thraustochytrids, dinoflagellate and/or *Mortierella*. More preferably, the LCPUFA-containing biomaterial for lipid extraction is selected from the group including *Schizochytrium*, *Thraustochytrium* and/or *Cryptocodinium* (preferably, *Cryptocodinium cohnii*) biomass or oilseeds from genetically modified plants containing genes from *Schizochytrium* or *Thraustochytrium* 10 and/or *Cryptocodinium* (preferably, *Cryptocodinium cohnii*).

Preferably, the initial lipid composition is predominantly made up of neutral lipids. Preferably, the initial lipid composition comprises at least 50% neutral lipids, preferably, at least 60% neutral lipids, preferably, at least 75% neutral lipids, preferably at least 85% neutral lipids and preferably at least 90% neutral lipids. Preferably, the neutral 15 lipid predominantly comprises triglyceride. Preferably, the initial lipid composition comprises at least 50% triglyceride, preferably, at least 60% triglyceride, preferably, at least 75% triglyceride and preferably at least 85% triglyceride. The foregoing percentages in this paragraph refer to weight percentages. Preferably, the concentration of the desired LCPUFA is greater in the resulting product than in the initial lipid 20 composition.

Preferred polar solvent:lipid ratios, based on weight, for the extraction or winterization process are from about 1:10 to about 20:1; more preferably from about 1:8 to about 10:1, preferably from about 1:5 to about 5:1, and preferably from about 1:2 to about 2.5:1. Preferably the contact time between the polar solvent and lipid is from about 25 0.5 to about 12 hours, preferably from about 2 to about 6 hours, and preferably about 4 hours. Preferably, if a nonpolar lipid is used, the residual nonpolar lipid is from about 0 to about 4 weight percent, and preferably from about 1 to about 4 weight percent.

Preferably the temperature for the: (i) cold extraction process, (ii) extraction followed by chilling and filtration/centrifugation, (iii) extraction, filtration/centrifugation 30 of cellular debris, followed by chilling and filtration/centrifugation; and (iv) chilling conditions for solvent winterization or polishing steps is from the solidification point of the lipid to the melting point of the undesirable component (e.g. trisaturated glycerides), more preferably from about -20°C to about 50°C, more preferably from about -5 °C to about 20 °C, more preferably from about -5°C to about 5°C, more preferably about 0 °C.

Other preferred attributes of the process include the selective recovery of only LCPUFA-containing triglycerides at the expense of trisaturated glycerides and other components that are relatively insoluble in cold acetone including phosphatides, wax esters, saturated fatty acid containing sterol esters, sterols, squalene, hydrocarbons and the like. By selectively recovering only the LCPUFA-containing triglyceride at the expense of these undesirable components allow the possibility of eliminating or reducing additional downstream purification steps (such as winterization, refining, and bleaching).

EXAMPLE 1

10 SUMMARY

A sample of DHA-rich lipid obtained from *Schizochytrium* (Sample 1, unwinterized lipid, a.k.a. "high melt") and an isolated sediment from another DHA-rich lipid obtained from *Schizochytrium* (Sample 2) were analyzed to determine the nature of the solid phase (Sample 1) and the floc/sediment (Sample 2).

15 Unwinterized lipid Sample 1 produced at plant scale (a semi-solid at ambient temperature) was dissolved in 4 volumes of cold acetone and mixed. A solid white powder (approximately 7% by weight) was isolated by filtration through a glass fiber filter. The solid white powder had a melting temperature of 52.4-53.5°C, was shown to be triglycerides (based on a single spot by thin layer chromatography (TLC)), and 20 contained predominantly myristic (26%) and palmitic acids (66%) when analyzed by GLC. This high melting triglyceride fraction contains saturated fatty acids with very little DHA/DPA. The isolated lipid fraction (91% by weight) was an orange-colored liquid at room temperature and contained 41.0% DHA and 16.0% DPA. DHA and DPA were enriched by approximately 8% compared to the starting fatty acid profile of Sample 1 – 25 this is a true "purification" of DHA and DPA.

Another DHA-rich reprocessed lipid from *Schizochytrium* contained an obvious floc-like material (haze) when stored for a period of days at ambient temperature. The floc was isolated by centrifugation. The floc/sediment ("Sample 2 sediment") was dissolved in 10 volumes of cold acetone, mixed and filtered. Approximately 15% by 30 weight of a solid white powder was isolated by filtration through a glass fiber filter. The solid white powder had a melting temperature of 50.1-51.4°C and was shown to be triglycerides (based on single spot by TLC) containing predominantly myristic (29%) and palmitic acids (59%). This is a high melting triglyceride fraction containing saturated fatty acids with little DHA/DPA. The isolated lipid fraction (85% by weight) was a clear,

orange-colored liquid at room temperature and contained 41.1% DHA and 16.3% DPA. The floc formation in reprocessed lipid from *Schizochytrium* is believed to result from a high melting triglyceride, containing myristic and palmitic fatty acids, which crystallizes from lipid upon standing.

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EXPERIMENTAL

General - A sample of DHA-rich lipid from Sample 1 (250g bottle) was pulled from frozen storage. This is a sample of unwinterized lipid. The sample was allowed to warm to ambient temperature and used as is.

10 Sediment (Sample 2) was isolated from DHA-rich lipid using a lab centrifuge. The DHA-rich lipid was a reprocessed lot of lipid that contained a visible floc when left to stand at ambient temperature. The floc was isolated by centrifuging the sample and decanting the liquid fraction from the sediment. The liquid fraction remained clear at ambient temperature; therefore the floc was believed to be present in the isolated 15 sediment.

20 *Acetone Winterization* - Unwinterized lipid (Sample 1) and sediment isolated from reprocessed lipid (Sample 2) were fractionated using an acetone winterization procedure. The sediment and unwinterized sample were dissolved in excess cold acetone (ice/water bath temperature) and mixed to dissolve and suspend lipid components. The solution/suspension was immediately filtered through a glass fiber filter under vacuum. The filter paper and the contents remaining on the paper were washed with small amounts 25 of cold acetone. The contents of the filter paper were air dried and weighed. The lipid/acetone fraction was concentrated under vacuum to afford neat lipid and weighed.

25 *TLC* – TLC was performed to determine lipid class composition using silica gel 60 plates. The developing solvent system consisted of a 90:10:1 mixture of petroleum ether: ethyl ether: acetic acid. The R_f of the spots were compared to those listed in “Techniques in Lipidology” by Morris Kates.

30 *Melting point determination* – Melting points were determined using a lab constructed melting point apparatus.

Infrared spectrometry – Infrared spectra were obtained using a Perkin Elmer 283B Infrared Spectrometer. Liquid fractions were analyzed neat. Solid fractions from acetone winterization were analyzed in chloroform.

Fatty Acid Methyl Esters (FAMEs) – Aliquots of DHA-rich lipid Sample 1, Sample 2 (reprocessed) along with acetone winterization fractions were transesterified

using anhydrous HCl in methanol following procedures for determining the free fatty acid profile, from C12 to C22:6. All FAME preparation and GLC work were completed. FAME's were identified and quantified using NuChek Prep analytical reference standard 502 using an internal standard (C19:0) to determine empirical response factors.

5 *Gas-liquid chromatography* - Gas-liquid chromatography of methyl esters was performed using a Hewlett-Packard Model 6890 Series II gas-liquid chromatograph equipped with a Hewlett-Packard autosampler, ChemStation software, a 30 m x 0.32mm SP-2380 capillary column (Supelco), and a flame-ionization detector. The oven temperature was held at 120°C for 3 min, programmed to 190°C at 5°C/min, held at
10 190°C for 1 min, programmed to 260°C at 20°C/min, and then held for 3 minutes at 260°C. The injector temperature was set at 295°C and the detector temperature was set at 280°C. Helium was used as a carrier gas and a split injection technique was employed.

RESULTS

15 **DHA-rich Lipid Sample 1**

A sample of unwinterized DHA-rich lipid (250g bottle) was pulled from frozen storage, Sample 1. This sample remained semi-solid at ambient temperature and can be technically referred to as a "fat", not an "oil". An aliquot (14.44 g) of the fat was transferred to an Erlenmeyer flask and 60 ml of cold acetone (ice/water bath) was added.
20 The flask was swirled to dissolve/suspend the fat components and immediately filtered through a glass fiber filter under vacuum. A solid white fraction remained on the filter paper and was washed with a few milliliters of cold acetone and dried. The solid white fraction was isolated in a 6.3% yield (0.91g starting from 14.44g fat).

The lipid/acetone fraction resulting from filtration was concentrated by rotary evaporation to afford 13.13 g of an orange-colored liquid material (liquid at ambient temperature). This resulted in a 91% overall recovery; therefore approximately 2% of material was lost at bench scale.

30 The solid white fraction and the lipid fraction isolated after "acetone winterization" were analyzed by TLC to determine lipid composition. The solid white fraction was shown to be triglycerides based on TLC (one spot with an R_f corresponding to a triglyceride was observed). Many spots were observed by TLC upon spotting and developing the lipid fraction. The R_f of the spots was consistent with lipid components

comprising squalene, steryl esters, triglycerides, and sterols (all tentative assignments). No further analysis of lipid class composition was performed.

The solid white fraction isolated after acetone winterization had a melting point range of 52.4-53.5°C.

5 The solid and liquid fraction isolated after acetone winterization were transesterified to methyl esters and the methyl esters were analyzed by gas-liquid chromatography. The complete profile of FAME's for both the solid and liquid fraction isolated by acetone winterization along with unwinterized DHA-rich fat (Sample 1) is shown in Table 1. As is evident, the solid fraction contained very little DHA (2.4%) and
10 DPA (0.9%) with methyl myristate (26%) and methyl palmitate (66%) as the predominant fatty acids. The liquid fraction isolated after acetone winterization contained myristate (8.3%), palmitate (23.1%), DPA (16.0%), DHA (41.0%) along with other minor fatty acids. When this profile is compared to that of the starting unwinterized lipid, an enrichment of the DHA of approximately 8% is seen, consistent with the removal of the
15 predominantly trisaturated glyceride component. This represents a purification step.

DHA-rich Lipid Sediment (Sample 2)

20 The sediment that was produced from re-refined lipid was completely miscible in hexane and not miscible in methanol. When small quantities of acetone were added to the sediment, a white precipitate formed which separated from the liquid, yellow-colored lipid/acetone phase. Based on these dissolution tests, acetone fractionation was used to isolate the white powder.

25 An aliquot (1.11 g) of sediment was transferred to an Erlenmeyer flask and 10 ml of cold acetone (ice/water bath) was added. The flask was swirled to dissolve/suspend the fat components and immediately filtered through a glass fiber filter under vacuum. A solid white fraction remained on the filter paper and was washed with a few milliliters of cold acetone and dried. The solid white fraction was isolated in a 15% yield (0.17g starting from 1.11g sediment).

30 The lipid/acetone fraction resulting from filtration was concentrated by rotary evaporation to afford 0.94 g of an orange-colored liquid material (liquid at ambient temperature). This resulted in an 85% overall recovery.

The solid white fraction and the lipid fraction isolated after acetone fractionation were analyzed by TLC to determine lipid composition. The solid white fraction was shown to be triglycerides based on TLC (one spot with an R_f corresponding to a

triglyceride was observed). Many spots were observed by TLC upon spotting and developing the lipid fraction. The R_f of the spots was consistent with lipid components comprising squalene, steryl esters, triglycerides, and sterols (all tentative assignments). No further analysis of lipid class composition was performed.

5 The solid white fraction isolated after acetone winterization had a melting point range of 50.1-51.4°C.

The solid and liquid fraction isolated after acetone winterization were transesterified to methyl esters and the methyl esters were analyzed by gas-liquid chromatography. The complete profile of FAME's for both the solid and liquid fraction 10 isolated by acetone winterization along with Sample 2 sediment is shown in Table 1. As is evident, the solid fraction contains very little DHA (6.4%) and DPA (2.6%) with methyl myristate (29%) and methyl palmitate (59%) as the predominant fatty acids. The liquid fraction isolated after acetone winterization contains myristate (8.4%), palmitate (23.2%), DPA (16.3%), DHA (41.1%) along with other minor fatty acids.

15

Table 1. Fatty acid profile of unwinterized oil (Sample 1), Sample 2 sediment and fractions isolated from Sample 1 and Sample 2 sediment by acetone fractionation

FA Name	Unwinterized Sample 1	Isolated Solid Fraction	Isolated Liquid Fraction Sample 1	Sample 2 Sediment	Isolated Solid Fraction Sample 2 Sediment	Isolated Liquid Fraction Lot 21A Sediment
14:0	9.6	25.9	8.3	12.2	27.0	8.4
16:0	25.9	66.0	23.1	30.5	58.8	23.2
16:1	0.3	<0.1	0.3	0.3	0.2	0.3
18:0	0.7	1.8	0.6	0.7	1.5	0.6
18:4 n3	0.4	<0.1	0.4	0.3	<0.1	0.4
20:3 n6	0.4	0.2	0.4	0.3	0.2	0.5
20:4 n7	2.8	<0.1	2.6	1.8	<0.1	2.4
20:4 n6	0.9	<0.1	1.0	0.8	0.1	1.0
20:4 n3	0.8	<0.1	0.9	0.8	<0.1	0.9
20:5 n3	2.2	<0.1	2.3	1.9	0.3	2.3
22:4 n9	0.2	<0.1	0.1	0.2	<0.1	0.2
22:5 n6	14.7	0.9	16.0	13.6	2.6	16.3
22:6 n3	37.7	2.4	41.0	34.2	6.4	41.1

20

Comparative Example

Table 2, set forth below, represents a comparative prior method as shown in Comparative Figure 1 followed by Comparative Figure 2.

5

Table 2
Certificate of Analysis
(Schizochytrium Biomass)
Refined, Deodorized, Bleached (RDB) Winterized Schizochytrium oil after
antioxidants addition

10

Table 2A

	Specification	Result	Method Reference
Peroxide Value, meq/kg	Maximum 3.0	0.42	AOCS Cd 8-53
Free Fatty Acids, %	Maximum 0.25	0.06	AOCS Ca 5a-40
Moisture and volatiles, %	Maximum 0.05	0.03	AOCS Ca 2d-25
Trace Metals, ppm			POS AS.SOP-103
Lead	Maximum 0.20	<0.20	
Arsenic	Maximum 0.20	<0.20	
Iron	Maximum 0.20	0.04	
Copper	Maximum 0.05	<0.05	
Mercury	Maximum 0.20	<0.20	
DHA, % of FAME, wt/wt	Minimum 32.0	43.5	POS AS.SOP-104
DHA, mg/g of oil	Minimum 300	397.3	POS AS.SOP-104
Residual Hexane, ppm	Maximum 10	<1.0	AOCS Ca 3b-87

Table 2B

	Specification	Value	Method Reference
Neutral oil, %	N/A	99.69	
p-Anisidine Value	N/A	0.74	AOCS Cd 18-90
Colour, 1.0" Lovibond (PFX 990 AOCS)	N/A	70.0Y 7.1R	AutoTintometer Colour
Colour, Gardner Scale, (1 cm)	N/A	12.3	
β -Carotene (PFX990), ppm, (0.01 cm)	N/A	276.41	
Note: not true β -Carotene			
Unsaponifiables, %	N/A	2.24	AOCS Ca 6b-53
Insoluble Impurities, %	N/A	0.01	AOCS Ca 3-46
AOM, hr	N/A	7.66	AOCS Cd 12-57
Rancimat (80°C), Hr	N/A	22.7	
Spin test, % solids by volume, 20°C/24hrs after antiox addition	N/A	~ 0.2*	
Spin test, % solids by Vol, before antiox addition	N/A	zero	
Fatty Acid Composition (absolute), mg/g	N/A		POS AS.SOP-104
C12		2.6	
C14		69.4	
C14:1		0.8	
C15		3.1	
C16		187.8	
C16:1		4.4	
C18		4.6	
C18:1		7.2	

	Specification	Value	Method Reference
C18:2		3.6	
C18:3n6		2.3	
C18:4		3.0	
C20		1.2	
C20:4n6		7.4	
C20:4n3 AA		8.5	
C20:5n3 EPA		18.2	
C22		0.6	
C22:5n6† DPA		151.6	
C22:6n3 DHA		397.3	
C24		1.8	
C24:1		1.9	
Others		35.1	
Total, mg/g		912.4	
DHA, % of FAME		43.5	
Ascorbyl palmitate, ppm		224	
Tocopherols, ppm		1,760	

* ppte from Addition of Rosemary extract.

5 Table 3, set forth below, represents a process of the present invention, as set forth in Figure 5 followed by the bleaching, deodorizing and refining of Comparative Figure 2.

10 **Table 3**
Acetone Winterized Schizo oil
RDB Schizo oil after antioxidants addition
 (From Schizophyllum biomass)

Table 3A

	Specification	Result	Method Reference
-Peroxide Value, meq/kg	Maximum 3.0	1.32	AOCS Cd 8-53
Free Fatty Acids, %	Maximum 0.25	0.06	AOCS Ca 5a-40
Moisture and volatiles, %	Maximum 0.05	0.03	AOCS Ca 2d-25
Trace Metals, ppm			POS AS.SOP-103
Lead	Maximum 0.20	<0.20	
Arsenic	Maximum 0.20	<0.20	
Iron	Maximum 0.20	0.11	
Copper	Maximum 0.05	<0.05	
Mercury	Maximum 0.20	<0.20	
DHA, % of FAME	Minimum 32.0	42.8	POS AS.SOP-104
DHA, mg/g of oil	Minimum 300	385.5	POS AS.SOP-104
Residual Hexane, ppm	Maximum 10	<1.0	AOCS Ca 3b-87

Table 3B

	Specification	Value	Method Reference
Neutral oil, %	N/A	99.69	
p-Anisidine Value	N/A	1.08	AOCS Cd 18-90
Colour, 1.0" Lovibond (PFX 990 AOCS)	N/A	70.0Y 6.3R	AutoTintometer Colour
Colour, Gardner Scale, (1 cm)	N/A	12.0	
β -Carotene (PFX990), ppm, (0.01 cm)	N/A	228.0	
Note: not true β -Carotene			
Unsaponifiables, %	N/A	2.11	AOCS Ca 6b-53
Insoluble Impurities, %	N/A	0.01	AOCS Ca 3-46
AOM, hr	N/A	7.00	AOCS Cd 12-57
<u>Rancimat (80°C), Hr</u>	N/A	19.9	
Spin test, % solids by volume, 20°C/24hrs	N/A	0.2	
Fatty Acid Composition (absolute), mg/g	N/A		POS AS.SOP-104
C12		3.9	
C14		90.1	
C14:1		0.8	
C15		3.4	
C16		193.9	
C16:1		6.5	
C18		4.8	
C18:1		8.1	
C18:2		3.6	
C18:3n6		1.7	
C18:4		2.6	
C20		1.5	
C20:4n6		4.9	
C20:4n3 AA		7.7	
C20:5n3 EPA		12.5	
C22		0.8	
C22:5n6† DPA		129.7	
C22:6n3 DHA		385.5	
C24		1.9	
C24:1		1.6	
Others		34.5	
Total, mg/g		900.0	
DHA, % of FAME		42.8	
Ascorbyl palmitate, ppm		222	
Tocopherols, ppm		1940	

Example 3

A crude extract of *Schizochytrium* oil was subjected to a variety of winterization procedures in which a lipid composition was extracted from biomass with hexane. The

hexane was removed to produce a crude extracted oil having a residual amount of hexane.

The extracted oil was then extracted with acetone at a particular acetone/oil ratio and winterized at a particular temperature for a given amount of time. The % residual hexane, acetone/oil ratio, winterization temperature and winterization time were varied in

5 different experiments. The processes were evaluated in terms of filtration time, oil recovery and haziness after two weeks. The details of the experiments and the results are shown below in Table 4.

10 Table 4. The levels of tested variables and observations of acetone-winterized Schizochytrium oil

Experiment No.	Hexane %	Acetone/Oil Ratio	Winterization Temp. (C)	Winterization Time (H)	Filtration @ (sec)	Oil Recovery (%)	Haziness After 2 weeks
1	1	1.5	5	3	67	87.8	Clear
2	2	1	0	2	165	86.4	PPT
3	2	1	0	4	195	87.7	Clear
4	2	1	10	2	178	88.1	PPT
5	2	1	10	4	154	89.8	PPT
6	2	2	0	2	85	84.1	PPT
7	2	2	0	4	75	86.2	Clear
8	2	2	10	2	67	88.9	PPT
9	2	2	10	4	82	86.7	PPT
10	3	0.5	5	3	264	84.3	PPT
11	3	1.5	-5	3	102	83.4	Clear
12	3	1.5	5	1	87	85.5	PPT
13	3	1.5	5	3	109	85.4	Clear
14	3	1.5	5	3	123	86.3	Clear
15	3	1.5	5	3	82	87.5	Clear
16	3	1.5	5	3	110	87.9	Clear
17	3	1.5	5	5	117	86.6	PPT
18	3	1.5	15	3	255	94.8	PPT
19	3	2.5	5	3	73	87.2	PPT
20	4	1	0	2	262	87.5	Clear
21	4	1	0	4	115	91.2	PPT
22	4	1	10	2	245	83.7	PPT
23	4	1	10	4	375	86.7	PPT
24	4	2	0	2	52	88.4	PPT
25	4	2	0	4	80	89.3	PPT
26	4	2	10	2	92	86.8	PPT
27	4	2	10	4	83	88.7	PPT
28	5	1.5	5	3	86	87.1	PPT
Control					150	90.9	PPT*

Control: Hexane winterization (45:55, Oil:Hexane) at -3C for 5h

PPT – Precipitate observed after spin-test

15 *The hexane winterized sample showed PPT after filtration (the same day), an indication of incomplete crystallization. The recovery obtained in the lab would not be duplicated in

the plant as the thorough drying of the cake may not be achievable with the enclosed filters.

Typical recovery in plant is around 70-75%.

5 Table 5. The oil recovery, filtration time and analytical data of crude oil, hexane and acetone-winterized oils.

Observations/analysis	Crude oil	Plant-Hexane-winterized oil	Lab-Hexane-winterized oil	Acetone-winterized oil (Verification trail-1)	Acetone-winterized oil (Verification trail-2)
Oil recovery (%)		70%	90.9	86.9	85.3
Filtration @ (Sec)		-	150	158	114
Color (1" cell) (1 cm cell)	Too dark 70Y 11.2R	-	70Y 12.3R	Too dark 70Y 12R	Too dark 70Y 11.1R
Phosphorus (ppm)	474.3	-	474.0	271.6	144.3
Free fatty acids	0.53	-	0.49	0.52	0.43
PV (meq/kg)	0.00	-	1.82	3.32	4.27
Anisidine value	4.11	-	4.37	3.73	3.66
Fatty acid comp. (mg/g)					
C12:0	2.3	-		2.2	2.1
C14:0	67.2	-	2.1	58.5	58.9
C14:1	0.7	-	57.8	0.8	0.8
C15:0	3.3	-	0.7	3.1	3.2
C16:0	204.9	-	3.1	187.0	188.1
C16:1	3.3	-	185.2	3.6	3.5
C18:0	5.1	-	3.5	4.5	4.7
C18:1	3.9	-	4.5	4.0	4.0
C18:2	2.6	-	4.0	2.7	2.7
C18:3n6	2.3	-	2.7	2.5	2.6
C18:4	3.3	-	2.5	3.6	3.6
C20:0	1.2	-	3.5	1.0	1.0
C20:4n6	9.4	-	1.0	10.2	10.3
C20:4n3	8.0	-	9.7	8.5	8.6
C20:5n3	23.6	-	8.3	25.4	25.6
C22:0	0.6	-	24.9	0.5	0.6
C22:5n6	142.9	-	0.6	152.7	154.0
C22:6n3	351.1	369.0*	149.6	378.6	382.2
C24:0	1.9	-	369.0	1.6	1.6
C24:1	4.0	-	1.6	4.3	4.2
Others	35.2	-	4.1	37.9	38.4
Recovery of DHA		73%	37.5	93.8%	92.8%
			95.6%		

*The estimation of DHA recovery of Pilot Plant hexane -winterized oil is based on the past data of Schizo oil process

Table 6. The fatty acid composition of acetone-winterized wax.

Observations/ Analysis	Acetone-winterized wax (Verification trial-1)	Acetone-winterized wax (Verification trial-2)
Wax recovery (%)	13.1	14.7
Fatty acid comp. (mg/g)		
C12:0	2.8	2.6
C14:0	112.4	103.2
C14:1	0.4	0.4
C15:0	3.8	0.6
C16:0	303.4	282.6
C16:1	2.1	2.1
C18:0	8.6	8.7
C18:1	3.3	3.6
C18:2	1.3	1.7
C18:3n6	1.1	1.0
C18:4	1.5	1.4
C20:0	2.2	2.0
C20:4n6	4.9	4.6
C20:4n3	4.1	4.0
C20:5n3	11.9	11.9
C22:0	1.3	1.2
C22:5n6	76.8	75.7
C22:6n3	175.2	170.5
C24:0	3.8	3.5
C24:1	2.1	2.0
Others	16.6	18.1

CONCLUSIONS

5 Based on an analysis of the Sample 2 sediment, it is believed the floc is triglycerides containing predominantly myristic and palmitic acids. This is based on TLC, IR, and resulting FAME analysis by GLC. The triglycerides comprising the floc had a high melting temperature (50.1-51.4°C).

10 The high melting temperature of the isolated white powder, coupled with the triglyceride lipid class composition of this fraction, indicates that the winterization step employed during standard processing is not quantitatively removing "high melting" fractions from the lipid. Therefore, an additional "polishing" step is recommended to achieve clarity in the finished goods product.

15 To estimate the solid contribution of unwinterized lipid in Sample 1, an acetone winterization procedure was employed. A solid white fraction isolated from Sample 1 in 6-7% yield was shown to be triglycerides containing predominantly myristic and palmitic acids (>94% of the fatty acids in this triglyceride component were saturated fats). Palmitic and myristic acid are present in roughly a 2:1 ratio and, coupled with the narrow

range in melting temperature, suggest a defined structure to this triglyceride. Very little DPA and DHA were present in the solid triglyceride fraction. The isolated liquid fraction following acetone winterization contained 41.0% DHA (expressed as a percentage of total fatty acid methyl esters) compared to 37.7% DHA in the starting unwinterized lipid. This 5 is an approximate 8% enrichment of DHA, consistent with the removal of 7% trisaturated fatty acid glycerides.

Very little loss of DHA was shown in the bench scale acetone winterization process, indicating near quantitative recovery of DHA can be obtained during winterization.

10 Solid or solvent assisted winterization (acetone winterization demonstrated herein, however other solvent alternatives exist) offer the following possibilities and can be considered as processing options.

- (1) A true removal of high melting, solid material can be accomplished.
- (2) The solid material is mainly trisaturated fatty acid glyceride (>94% 15 saturated fatty acids) with very little DHA (2.4%).
- (3) As an example calculation, starting from 1,000 kg's of DHA in crude lipid, an approximate loss of 2 kg's of DHA would be encountered during acetone winterization ($1,000 \times 0.07 \times 0.024$). This is approximately a 0.2% recovery loss of DHA on an absolute weight basis.
- (4) A clear liquid remains following winterization, with enrichment of DHA 20 compared to the starting unwinterized lipid fatty acid profile.
- (5) Solvent assisted winterization can be used to achieve DHA purification.
- (6) Because of the high melting temperature of the trisaturated fatty acid glyceride component (>50°C), traditional low temperature chilling conditions may 25 not be required.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those 30 skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for purifying a lipid composition comprising predominantly neutral lipid, wherein said lipid composition contains at least one long chain polyunsaturated fatty acid (LCPUFA) obtained from a microbial biomass having at least 50% triglycerides; from 0 to 4% non-polar solvent, and at least one other compound, said process comprising:
 - (a) contacting said lipid composition with a polar solvent, wherein said other compound is less soluble than said LCPUFA in said polar solvent;
 - (b) maintaining said lipid composition at a temperature range effective to precipitate at least a portion of said other compound; and
 - (c) removing at least a portion of said other precipitated compound from said lipid composition to form a lipid product.
2. The process of claim 1, wherein said lipid composition comprises at least 85% neutral lipid.
3. The process of claim 1 or 2, wherein said neutral lipid comprises triglyceride.
4. The process of any one of claims 1 to 3, wherein said polar solvent is selected from the group consisting of acetone, isopropyl alcohol, methanol, ethanol, ethyl acetate and mixtures thereof.
5. The process of any one of claims 1 to 4, wherein said polar solvent comprises acetone.
6. The process of any one of claims 1 to 5, wherein said temperature range is from about -20°C to about 50°C.
7. The process of any one of claims 1 to 6, wherein said temperature range is from about -5°C to about 20°C.

8. The process of any one of claims 1 to 7, wherein said temperature range is from about -5°C to about 5°C.

9. The process of any one of claims 1 to 8, wherein said temperature is about 0°C.

10. The process of any one of claims 1 to 9, wherein said LCPUFA is selected from the group consisting of arachidonic acid (ARA), omega-6 docosapentaenoic acid (DPA(n-6)), omega-3 docosapentaenoic acid (DPA(n-3)), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

11. The process of any one of claims 1 to 10, wherein said other compound is selected from the group consisting of trisaturated glycerides, phosphorus-containing materials, wax esters, saturated fatty acid containing sterol esters, sterols, squalene, and hydrocarbons.

12. The process of any one of claims 1 to 11, wherein said other compound is selected from the group consisting of trisaturated glycerides, phosphatides and wax esters.

13. The process of any one of claims 1 to 12, wherein said other compound is selected from the group consisting of trisaturated glycerides of lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) fatty acids and mixtures thereof.

14. The process of any one of claims 1 to 13, wherein said LCPUFA is obtained from a source selected from the group consisting of thraustochytrid biomass, dinoflagellate biomass, *Mortierella* biomass, and oilseeds from genetically modified plants containing genes from thraustochytrids, dinoflagellates or *Mortierella*.

15. The process of any one of claims 1 to 14, wherein said LCPUFA is obtained from the group comprising *Schizochytrium*, *Thraustochytrium* or

Cryptothecodium cohnii biomass or oilseeds from genetically modified plants containing genes from *Schizochytrium* or *Thraustochytrium*.

16. The process of any one of claims 1 to 15, wherein the polar solvent:lipid composition ratio is from about 1:10 to about 20:1.

17. The process of any one of claims 1 to 16, wherein the polar solvent:lipid composition ratio is from about 1:8 to about 10:1.

18. The process of any one of claims 1 to 17, wherein the polar solvent:lipid composition ratio is from about 1:5 to about 5:1.

19. The process of any one of claims 1 to 18, wherein the polar solvent:lipid composition ratio is from about 1:2 to about 2.5:1.

20. The process of any one of claims 1 to 19, wherein the polar solvent:lipid composition ratio is about 1:1.

21. The process of any one of claims 1 to 20, wherein the time of contact between said polar solvent and said lipid composition is from about 0.5 to about 12 hours.

22. The process of any one of claims 1 to 21, wherein the time of contact between said polar solvent and said lipid composition is from about 2 to about 6 hours.

23. The process of any one of claims 1 to 22, wherein the time of contact between said polar solvent and said lipid composition is about 4 hours.

24. The process of any one of claims 1 to 23, wherein the lipid composition comprises at least 50% neutral lipid by weight.

25. The process of any one of claims 1 to 24, wherein the lipid composition comprises at least 50% triglyceride by weight.

26. The process of any one of claims 1 to 25, wherein the DHA content of the lipid composition is enriched to at least 41% DHA by weight.

27. The process of any one of claims 1 to 26, wherein said lipid composition does not contain an extraction solvent prior to contacting said lipid composition with said polar solvent.

1/5

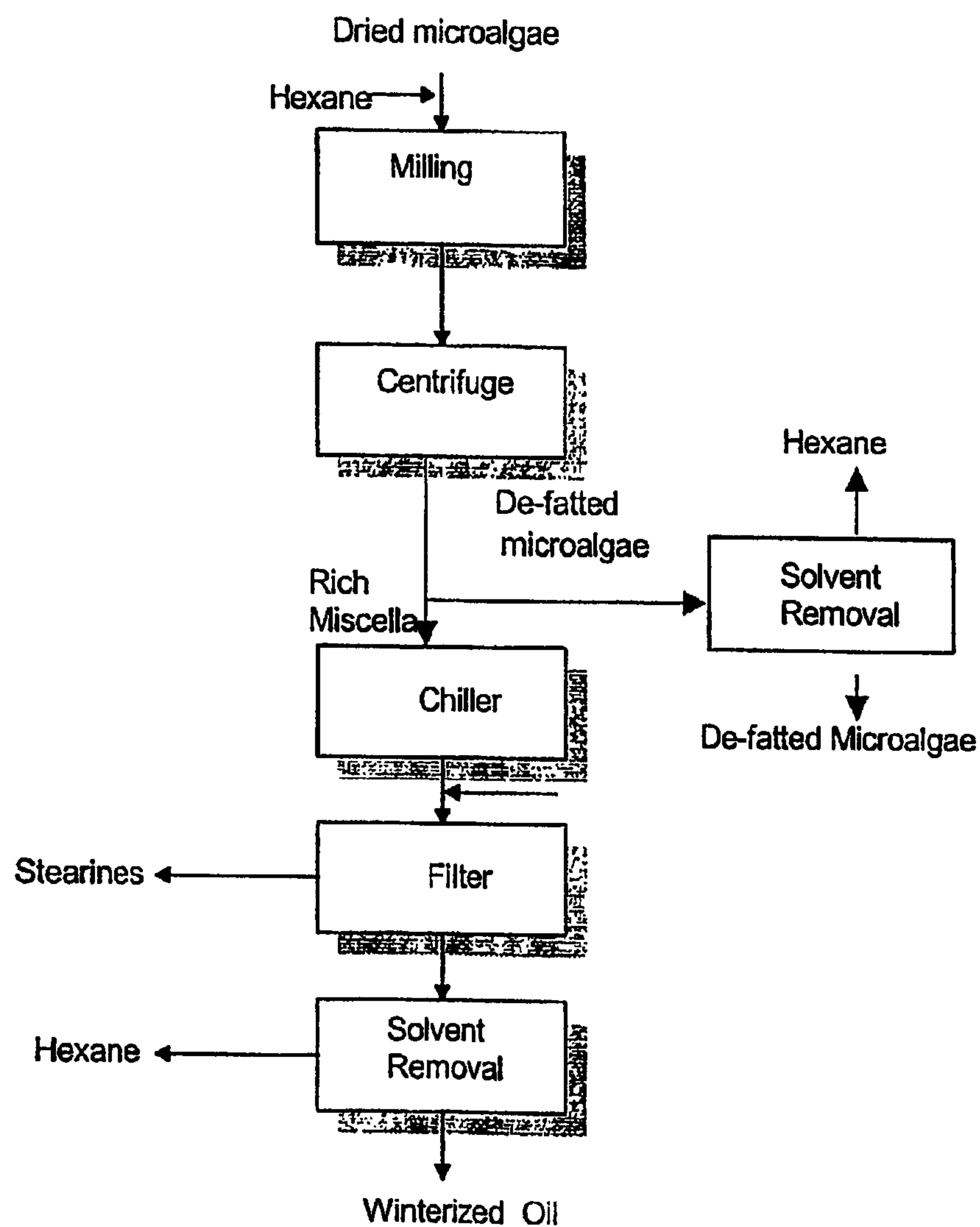


Figure 1. Overview of the DHA-rich lipid extraction process using hexane

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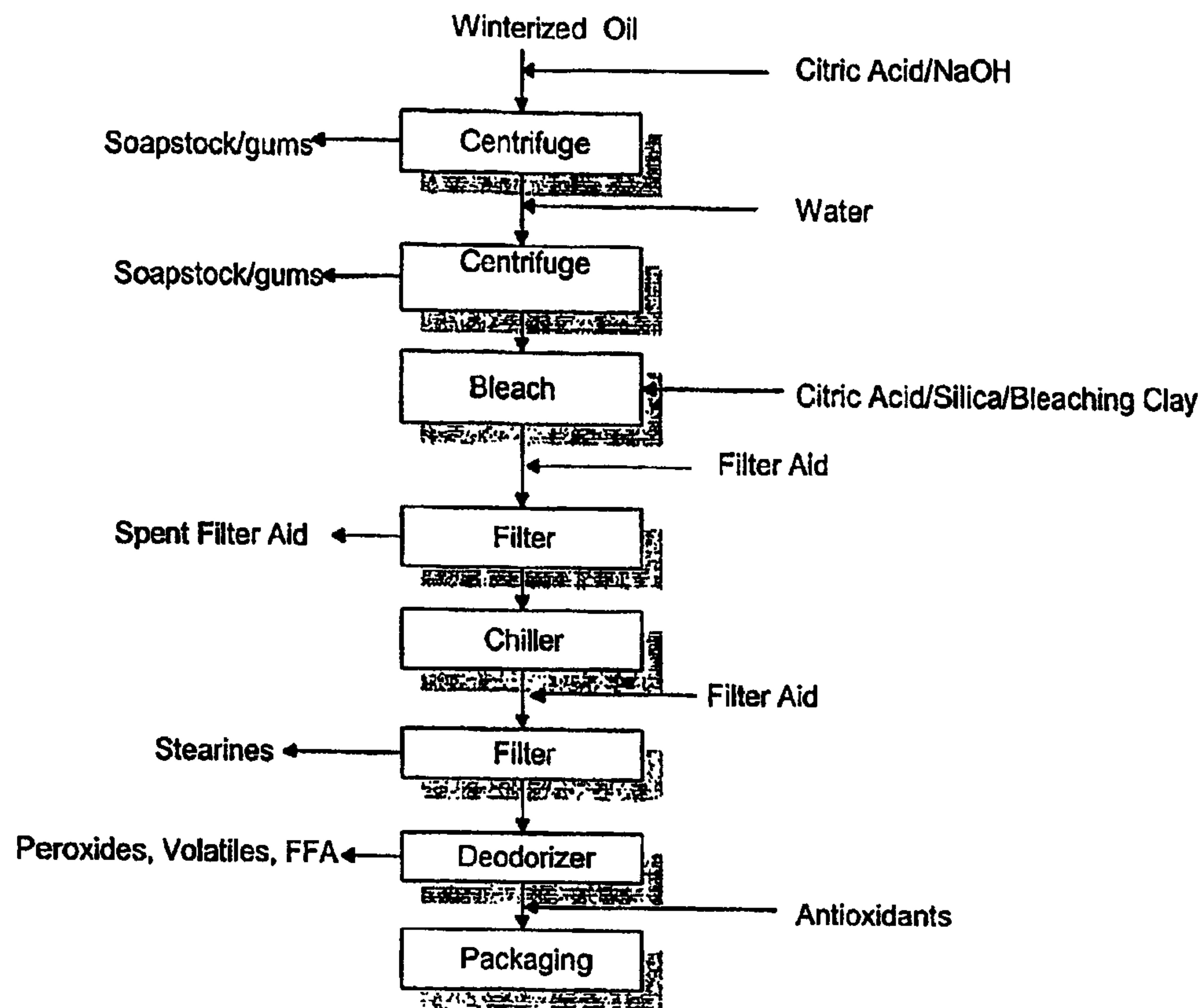


Figure 2. Overview of the DHA-rich lipid purification process

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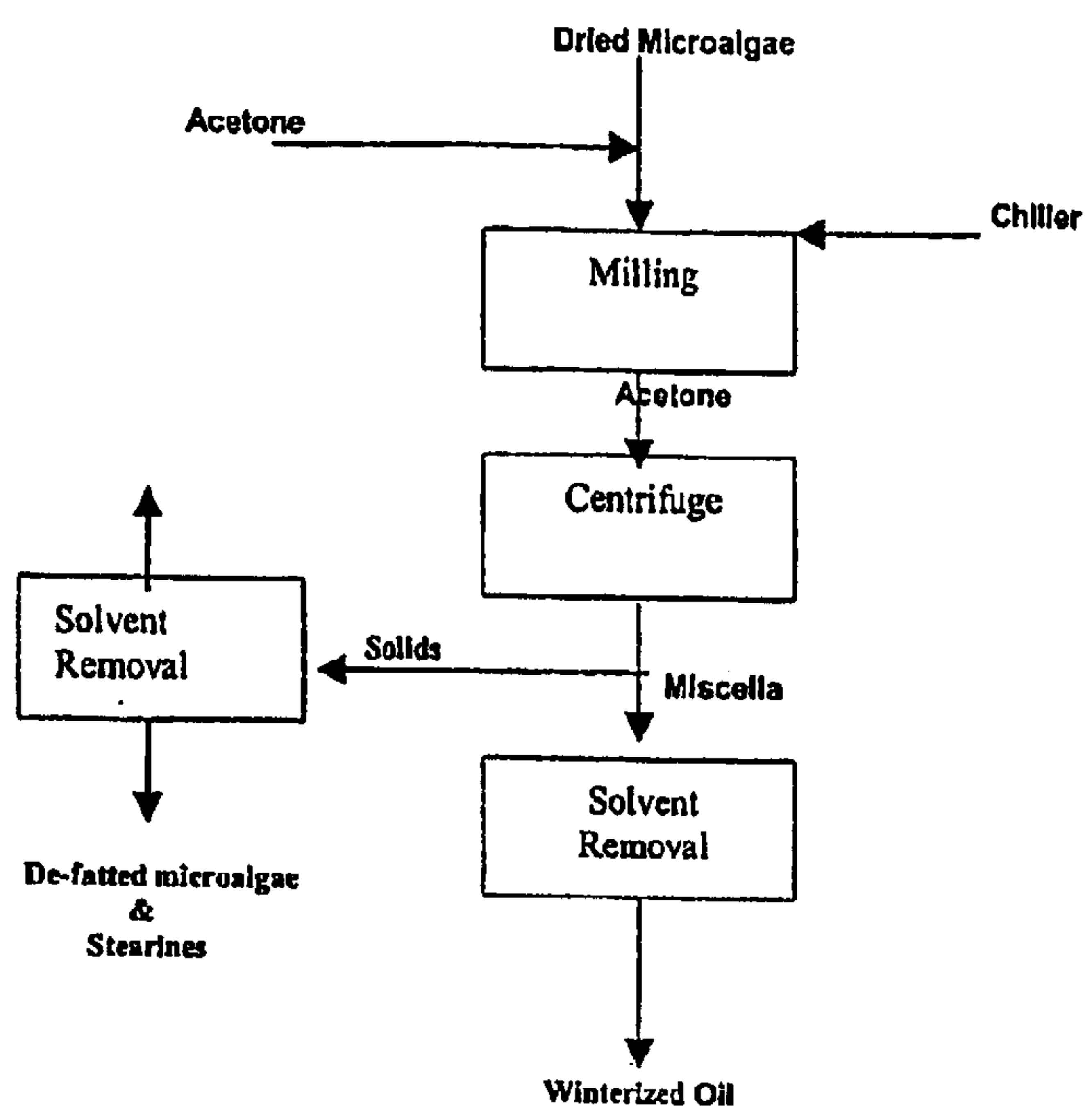


Figure 3. Overview of the DHA-rich lipid extraction process using acetone in one step

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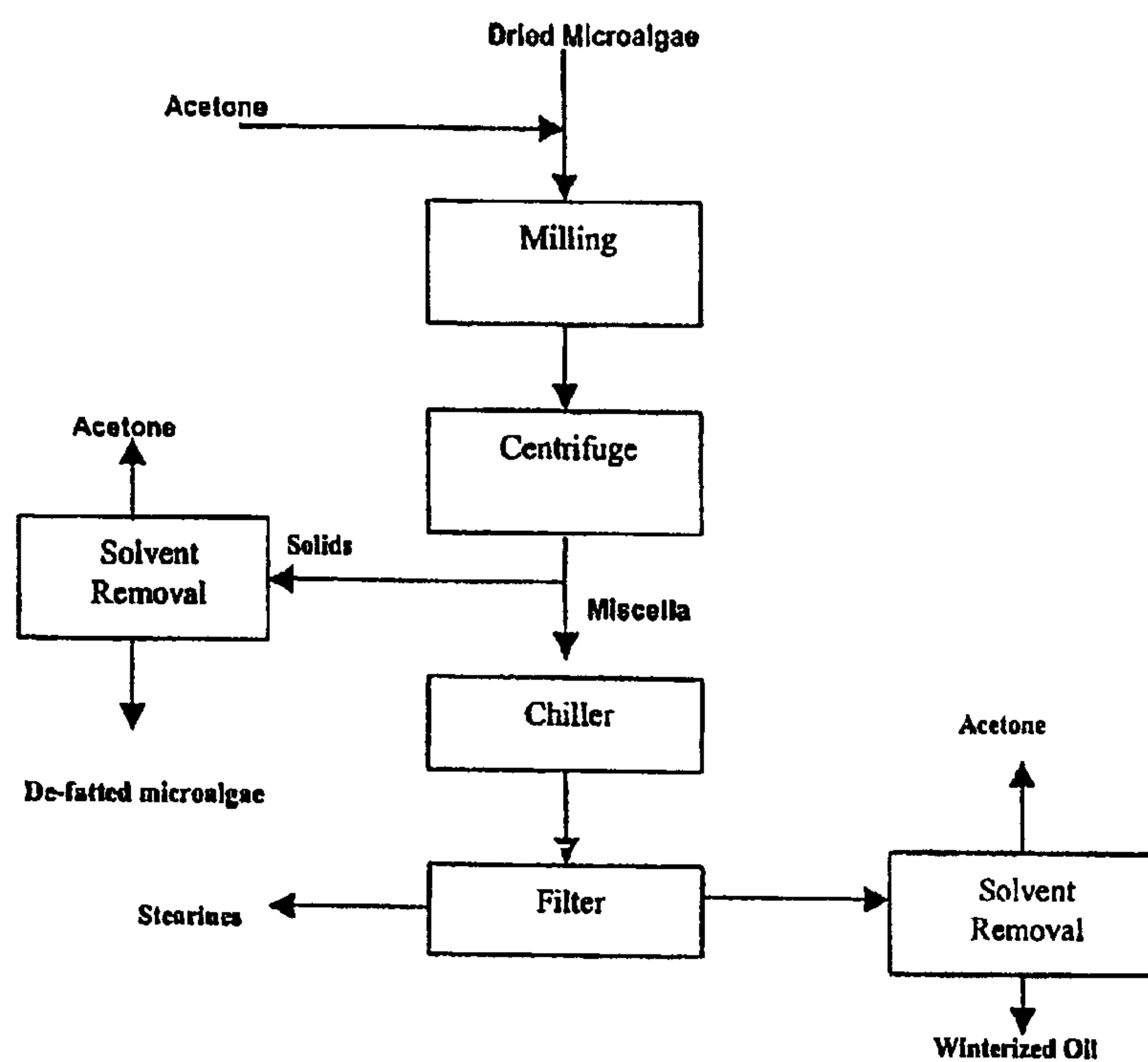


Figure 4. Overview of the DHA-rich lipid extraction process using acetone in two steps

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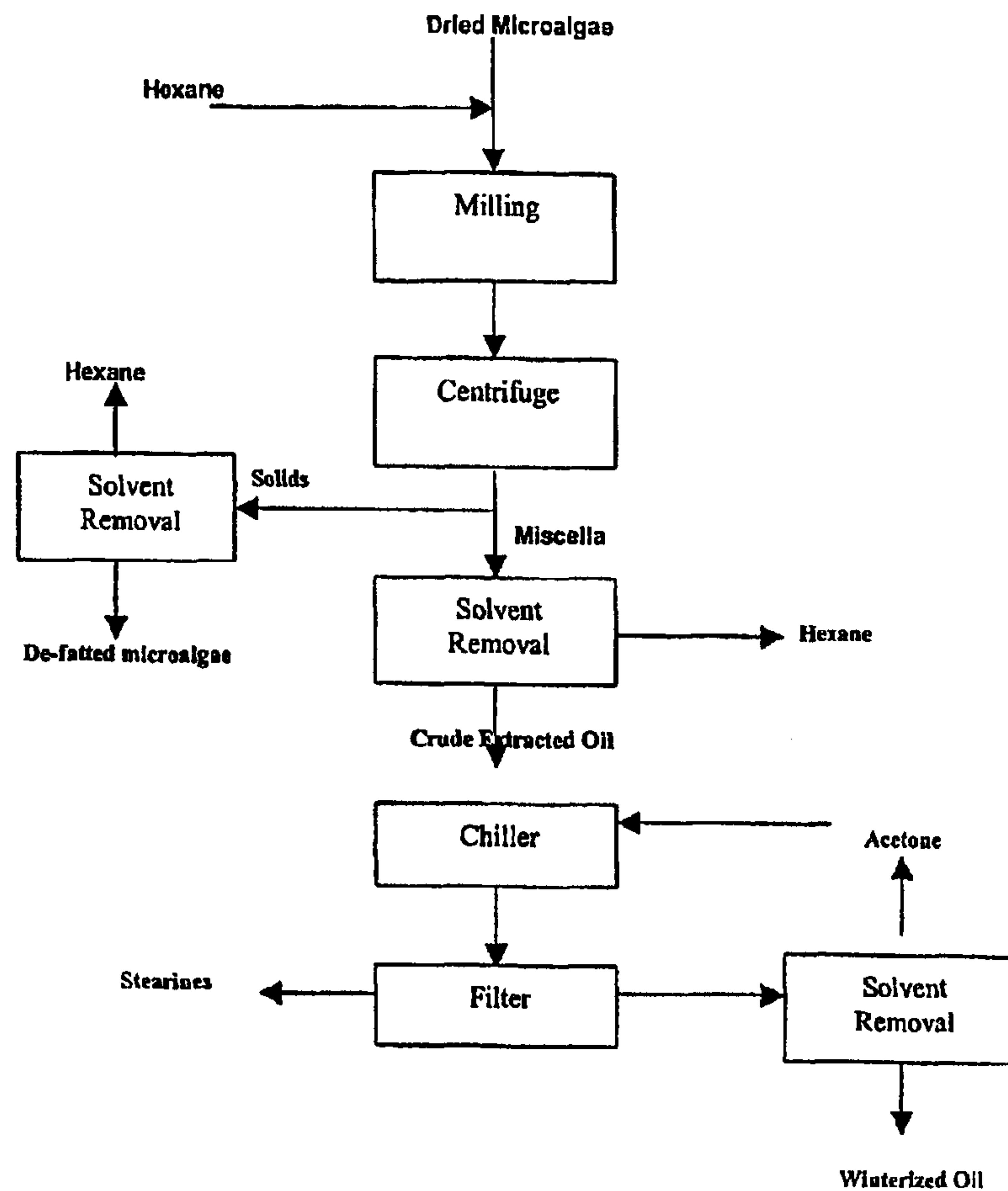
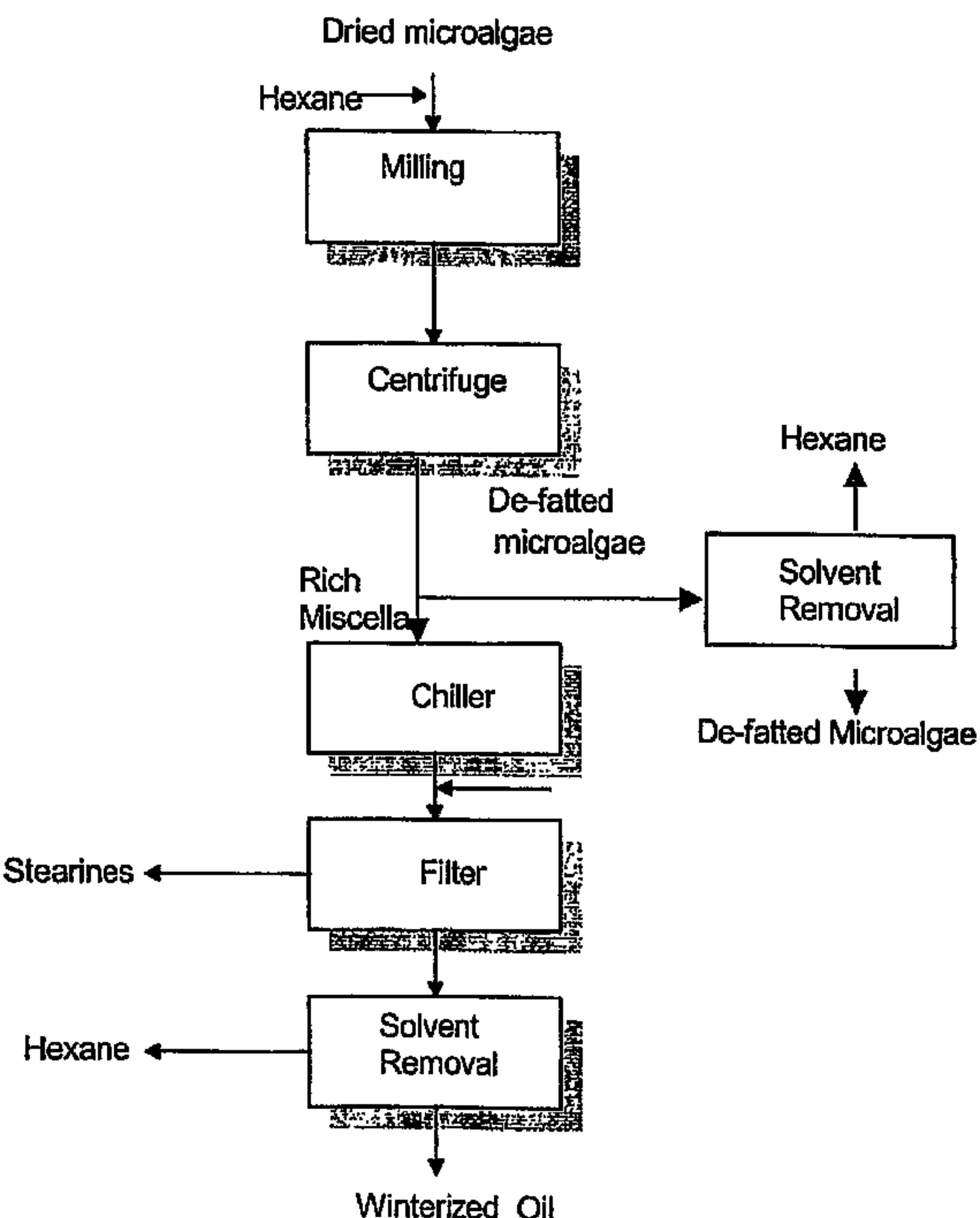


Figure 5. DHA-rich lipid hexane extraction process and acetone winterization



Overview of the DHA-rich lipid extraction process using hexane