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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2007 (01.11.2007)

PCT

(10) International Publication Number
WO 2007/123425 A1

(51) International Patent Classification:
C11B 7/00 (2006.01) *A23L 1/48* (2006.01)
C11B 1/10 (2006.01)

(21) International Application Number:
PCT/NZ2007/000088

(22) International Filing Date: 20 April 2007 (20.04.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
546682 20 April 2006 (20.04.2006) NZ

(71) Applicants and

(72) Inventors: **STEVENSON, David,** [GB/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **SCOTT, Dawn,** [NZ/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **CATCHPOLE, Owen John,** [NZ/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **MACKENZIE, Andrew,** [NZ/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **VYSSOTSKI, Mikhail** [RU/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **SVETASHEV, Vasily,** [RU/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **FENTON, Tina,** [NZ/NZ]; C/- Industrial Research Limited, Gracefield Research Centre, Gracefield Road, Lower Hutt (NZ). **MURNEY, Jason,** [NZ/NZ]; C/- Fonterra Research Centre, Dairy Farm Road, Palmerston North (NZ).

(74) Agents: **ADAMS, Matthew, D** et al.; A J Park, 6th Floor Huddart Parker Building, Post Office Square, P O Box 949, Wellington, 6015 (NZ).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/123425 A1

(54) **Title:** PROCESS FOR SEPARATING LIPID MATERIALS

(57) **Abstract:** The present application relates to processes for fractionating a feed material into soluble and insoluble components, by contacting a feed material and a solvent and subsequently separating the solvent containing the soluble components from the insoluble components, wherein the feed material comprises one or more of: at least 1% w/w phosphatidyl serine, at least 1% w/w sphingomyelin, at least 1% w/w cardiolipin, or at least 0.3% w/w gangliosides, and wherein the solvent comprises one or more C_1-C_3 monohydric alcohols and water (wherein the water content of the one or more alcohols is 0 to 40% v/v).

PROCESS FOR SEPARATING LIPID MATERIALS

FIELD OF INVENTION

This invention relates to fractionation processes. More particularly it relates to a process for fractionating a feed material containing phospholipids (including for example phosphatidyl serine, cardiolipin or sphingomyelin), gangliosides, or a combination thereof.

BACKGROUND

Phospholipids are major components of all biological membranes, and include phosphoglycerides (for example phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), cardiolipin (CL) and phosphatidyl serine (PS)); and sphingolipids such as sphingomyelin (SM).

Gangliosides (GS) are components in the cell plasma membrane which modulate cell signal transduction events. They are implicated as being important in immunology, brain function and neurodegenerative disorders.

Both phospholipids and sphingolipids are involved in cell signalling events leading to, for example, cell growth, cell differentiation, cell proliferation, and programmed cell death (apoptosis).

Reasonable levels of these components can be found in milk, soy products, eggs, animal tissues, marine animals, plants, micro-organisms and other sources. A well known source is the bovine milk fat globule membrane (MFGM) which is known to contain useful quantities of sphingomyelin, cerebrosides, gangliosides, phosphatidyl serine and other phospholipids (Jensen 2002).

Both phospholipids and sphingolipids have been implicated in having a number of health benefits, including brain health, sports nutrition, skin health, eczema treatment, anti-infection, wound healing, gut microbiota modifications, anti-cancer activity, alleviation of arthritis, improvement of cardiovascular health, and treatment of metabolic syndromes.

Alcohol-water mixtures have previously been applied to separation or enrichment of the phospholipids PC, PE and PI and/or to gangliosides. Wu & Wang (2004) describe the use of

aqueous ethanol and different temperature treatments to fractionate soybean lecithin. The conditions were optimized to produce high PC and high PI fractions. It was found that a higher oil (neutral lipid) content significantly increases the yield of the PC fraction, but significantly decreases the yield, purity and recovery of the PI fraction. Stirring of the lecithin-ethanol mixture for one hour followed by centrifugation resulted in the formation of a PC-enriched fraction (supernatant) and a PI-enriched fraction (precipitate). After removal of the solvent, the PI fraction was treated with acetone to remove neutral lipids. Aqueous ethanol was used in concentrations of 84%, 89%, 95% and 99% at 5:1 solvent to lecithin ratio. The optimal moisture content of lecithin-ethanol mixtures was found to be 24% which was achieved by using a 95% ethanol-to-lecithin ratio of 5:1. Yield, purity and recovery of PC fraction increased with increasing temperature, with maximum purity achieved at 40 °C. The optimal solvent-to-sample ratio was 6:1 (PC yield- and recovery wise) or 10:1 (for PI). Maximum purity of PC was achieved when fractionated twice, but the purity of PI fractions decreased with increasing number of fractionations. Optimal fractionation required a feed sample oil content of 40%. The use of higher temperature fractionation, followed by applying a lower temperature after fractionation, increased the purity of PC fraction, although the yield and recovery of the PC fraction were low. A process for obtaining PS-, SM-, CL- or GS-enriched fractions was not disclosed.

The traditional approach used to obtain high PS fractions is not by enrichment of PS-containing mixtures, but rather by the use of phospholipase D, serine and PC or a phospholipid mixture containing PC (US 5,700,668 A, KR2003086128 A, and JP 2079990 A). Here, the phospholipase D enzyme is used to convert PC to PS.

In JP 2002241385 A, a mixture of phospholipids is dissolved in alcohol, followed by the addition of metallic salts to precipitate PS, while JP 3047192 A and JP 2805522 B2 disclose the use of centrifugal partition chromatography with an aqueous mixed solvent containing a saturated hydrocarbon, alcohol and ether to separate PE, PC and SM.

JP 8322472 A discloses a process for concentrating acidic lipids from plant (soybean) lecithin. Plant-derived lecithin, suitably soybean lecithin containing at least 40% phospholipids, is treated with a lower alcohol containing less than or equal to 15% water, and preferably less than or equal to 10% water, at over 30 °C, and preferably at 35-80 °C. The lower alcohol fraction is then removed. The phospholipids to lower alcohol ratio is preferably greater than 15 on a weight basis. In addition, the treatment is preferably repeated 3-5 times where each treatment uses a phospholipids-to-low alcohol ratio of greater than one

on a weight basis. The resulting acidic phospholipid concentrate preferably contains more than 60% PI and phosphatidic acid (PA). JP 95151260 A and US 5,833,858 A describe a more elaborate approach wherein the first step comprises treating plant-derived lecithin with a solvent containing 85% to 100% of a lower alcohol and then eliminating the solvent fraction containing said lower alcohol to give a phospholipid mixture, and the second step comprises treating the said phospholipid mixture with an aqueous solution containing 75% or less of ethanol. The water-soluble components contained in the phospholipid mixture obtained in the first step are efficiently removed in the second step. Thus a lipid mixture containing a high concentration of the acidic phospholipids PI and PA can be relatively easily obtained from plant-derived lecithin. However, a process for concentrating PS, SM, CL or GS was not disclosed.

JP 10265485 A describes the use of ammonia-containing ethanol to prepare highly concentrated PA and PI mixtures, as well as PE and PC mixtures, from lecithin. A further, more elaborate process is disclosed in US 5,214,171 A for fractionating phosphatide mixtures into two or more fractions which are enriched in one or more of PC, PE, PI and PA by carrying out extraction steps using an alcoholic solvent in which the solubilities of PC, PE and PA are controlled by suitably adjusting the acidity of the solvent, the pH being adjusted to above 8 for solubilizing PC and PE and to below 5 for solubilizing PA. PI is substantially insoluble in the solvent used in the process and thus is mainly recovered in the extraction residue. Further parameters influencing the solubility of the components of the phosphatide mixtures to be fractionated are water content of the alcoholic solvent, temperature and choice of bases and acids for adjusting the pH. For further separating fractions rich in PC and PE di- or trivalent metal salt solutions were used. A process for concentrating PS, SM, CL or GS was not disclosed.

To produce SM-enriched fractions, mixtures containing 5-15% of SM were dissolved in alcohol (optionally containing water) and the solution was concentrated, followed by extraction with aliphatic hydrocarbons and precipitation with aliphatic ketones (WO 2000/45828 A1). A somewhat similar approach was disclosed in WO 94/18289 A1, where phospholipids are mixed with lower aliphatic alcohols (methanol, ethanol, propanol, butanol) and essentially non-polar solvents (hydrocarbons or chloroform), the non-polar phase is collected and SM is precipitated by addition of ketone or methyl or ethyl acetate. These processes have the disadvantage of using multiple solvents to achieve the desired separation.

In small-scale laboratory practice, butanol-water- diisopropylether partitioning is an effective way to prepare GS-enriched fractions (Ladish & Gillard 1985). This method is applicable to the total lipid extracts of plasma, cells, or animal tissues. Partitioning of the dried total lipid extract is performed in a three-component solvent system consisting of diisopropyl ether, 1-butanol, and 50 M aqueous NaCl (6/4/5, v/v/v). Gangliosides partition nearly quantitatively into the lower aqueous phase, and other lipids into the upper organic phase resulting from the mixture of these three solvents. The ganglioside-containing aqueous phase is then freed of salts and other low-molecular-weight impurities by gel filtration.

Commercial scale production of gangliosides often involves heating ganglioside containing raw materials, like buttermilk or whey protein concentrate, under alkaline conditions (e.g. US 5,831,079, US 5,795,980, JP 93035155 B, JP 3615798 B2). It should be noted that under these conditions ganglioside GD3 is hydrolysed and forms ganglioside GM3. Other commonly used approaches include ultrafiltration (e.g. JP 3176698 B2, WO 91/07417), column chromatography (e.g. DE4221190 A1, CA 2002155 A, IT 1252310 B) or both (e.g. JP 2207090 A, CN 1379034 A).

A composition containing at least 4% gangliosides was produced by adding ethanol to milk or milk products to achieve a final ethanol concentration of 30-70 vol% and recovering the precipitate; then by adding ethanol to the precipitate to give an ethanol concentration of 70-90 vol%, removing the protein precipitate, and membrane filtering the ethanol fraction (US 5,844,104).

Preparation of ganglioside-enriched compositions is also described in US 6,265,555. This patent claims a method of manufacturing a composition containing ganglioside from milk or a milk-derived material (butterserum, buttermilk, whey, and whey protein concentrate), comprising the steps of: dispersing a material containing ganglioside in an ethanol solution to make the concentration of ethanol 60-95%; heating said dispersion to a temperature of 50-90°C to dissolve ganglioside and simultaneously generate a first precipitate containing proteins; removing the first precipitate; cooling a supernatant excluding said first precipitate to a temperature lower than 0°C to generate a second precipitate containing ganglioside; and recovering the second precipitate.

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents or such sources of information is not to be construed as

an admission that such documents or such sources of information, in any jurisdiction, are prior art or form part of the common general knowledge in the art.

It is an object of this invention to provide a process for producing a product that contains desirable levels of particular phospholipids and/or gangliosides or at least to offer the public a useful choice.

SUMMARY OF INVENTION

Accordingly, the present invention provides a process for fractionating a feed material into soluble and insoluble components, comprising

(a) providing a feed material comprising one or more of:

(i) at least 1% w/w phosphatidyl serine,

(ii) at least 1% w/w sphingomyelin,

(iii) at least 1% w/w cardiolipin, or

(iv) at least 0.3% w/w gangliosides

(b) providing a solvent comprising one or more C_1-C_3 monohydric alcohols and water, wherein the water content of the one or more alcohols is 0 to 40% v/v

(c) contacting the feed material and the solvent and subsequently separating the solvent containing the soluble components from the insoluble components

(d) optionally separating the soluble components and the solvent.

Preferably the feed material comprises less than 10% protein, and less than 10% lactose. More preferably less than 10% of the feed material comprises protein and lactose.

Alternatively the feed material comprises greater than 1% phosphatidyl serine. Preferably the feed material comprises greater than 2% phosphatidyl serine. More preferably the feed material greater than 5% phosphatidyl serine.

Alternatively the feed material comprises greater than 1% sphingomyelin. Preferably the feed material comprises greater than 5% sphingomyelin. More preferably the feed material comprises greater than 15% sphingomyelin.

Alternatively the feed material comprises greater than 1% cardiolipin. Preferably the feed material comprises greater than 2% cardiolipin. Most preferably the feed material comprises greater than 5% cardiolipin.

Alternatively the feed material comprises greater than 0.3% gangliosides. Preferably the feed material comprises greater than 1% gangliosides. Most preferably the feed material comprises greater than 2% gangliosides.

The feed material of the present invention may be derived from terrestrial animals, marine animals, terrestrial plants, marine plants, or micro-organisms such as microalgae, yeast and bacteria. Preferably the feed material is derived from sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human.

Optionally the feed material is selected from: tissue, a tissue fraction, organ, an organ fraction, milk, a milk fraction, colostrum, a colostrum fraction, blood and a blood fraction.

Preferably the feed material is derived from dairy material, soy material, eggs, animal tissue, animal organ or animal blood. More preferably the feed material is selected from: a composition comprising dairy lipids, a composition comprising egg lipids, and a composition comprising marine lipids.

Most preferably the feed material used in the process of the present invention is a bovine milk fraction. Preferably the feed material is selected from: buttermilk, a buttermilk fraction, beta serum, a beta serum fraction, butter serum, a butter serum fraction, whey, a whey fraction, colostrum, and a colostrum fraction.

The feed material may comprise milk fat globule membrane.

The feed material may have been genetically modified.

Preferably, the feed material is in solid form. When solid, the feed material may be cryomilled before contact with the solvent.

Preferably the feed material used in the process of the present invention is cryomilled to achieve a mean particle size of 0.1-5mm before contact with the solvent.

The solvent of the present invention preferably comprises:

(a) an alcohol selected from: methanol, ethanol, n-propanol, isopropanol and mixtures thereof; and

(b) 0 – 40% v/v water

More preferably the solvent comprises between 0 and 20% v/v water. Most preferably the solvent comprises between 1 and 10% v/v water.

Preferably the alcohol is ethanol.

Preferably the solvent used in the process of the present invention comprises 95% aqueous ethanol.

Preferably the ratio of solvent to feed material in the process of the present invention is between 10:1 to 100:1. More preferably the ratio of solvent to feed material is between 20:1 to 60:1. Most preferably the ratio of solvent to feed material is between 25:1 to 40:1.

Preferably the process of the present invention is carried out at greater than 30°C. More preferably the process of the present invention is carried out at greater than 50°C. Most preferably the process of the present invention is carried out at greater than 55°C.

The process of the present invention may be carried out in a number of steps of decreasing or increasing temperatures.

Preferably the process of the present invention is carried out at less than or equal to 4°C. More preferably the process is carried out at less than or equal to 0°C.

Preferably step (c) of the process of the present invention is carried out for between 1-3 hours. Most preferably step (c) is carried out for 2 hours.

In the process of the present invention, the solvent and feed material are contacted once, the solvent and feed material may be contacted multiple times with the same batch of solvent, or the feed material may be contacted with more than one batch of solvent.

Preferably the solvent and feed material are contacted by using a packed bed of feed material with the solvent flowing through it.

Alternatively the solvent and feed material are mixed by agitation. Preferably the agitation is achieved by high shear mixing.

The invention also provides products produced by the process of the invention, both the insoluble components remaining after contact with the solvent (also referred to herein as the "residue"); and the soluble components that are dissolved in the solvent after contact with the feed material (also referred to herein as the "extract"). Where the feed material is contacted with more than one batch of solvent, or the solvent is cooled in a number of steps, there will be multiple "extract" products.

Preferably the product contains more sphingomyelin than the feed material.

Preferably the product comprises greater than 3% sphingomyelin. More preferably the product comprises greater than 10% sphingomyelin. Most preferably the product comprises greater than 15% sphingomyelin.

Preferably the product contains more phosphatidyl serine than the feed material

Preferably the product comprises greater than 5% phosphatidyl serine. More preferably the product comprises greater than 30% phosphatidyl serine. Most preferably the product comprises greater than 70% phosphatidyl serine.

Preferably the product contains more gangliosides than the feed material

Preferably the product comprises greater than 2% gangliosides. More preferably the product comprises greater than 4% gangliosides. Most preferably the product comprises greater than 6% gangliosides.

Preferably the product contains more cardiolipin than the feed material

Preferably the the product comprises greater than 5% cardiolipin. More preferably the product comprises greater than 10% cardiolipin. Most preferably the product comprises greater than 25% cardiolipin.

ABBREVIATIONS AND ACRONYMS

In this specification the following are the meanings of the abbreviations or acronyms used.

"PL" means phospholipids

"CL" means cardiolipin (diphosphatidyl glycerol)

"PC" means phosphatidyl choline

"PI" means phosphatidyl inositol

"PA" means phosphatidic acid

"PS" means phosphatidyl serine

"PE" means phosphatidyl ethanolamine

"SM" means sphingomyelin

"DHSM" means dihydrosphingomyelin

"GS" means gangliosides

"MFGM" means milk fat globule membrane

"N/D" means not detected

GENERAL DESCRIPTION OF THE INVENTION

The present invention is based on recognising that there is a difference in solubility of phospholipids in short-chain monohydric alcohol solvents. Using this solubility differential, the inventors have discovered that the insoluble phospholipids (including PS and CL) and GS may be fractionated from the soluble phospholipids (including SM).

A number of variables in this process can be manipulated to optimise the fractionation, as follows;

(1) Type of feed material.

A variety of feed materials may be used in the present invention.

For example, the feed material may be derived from sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human tissue, milk, colostrum, or blood. Alternatively the feed material may be derived from soy or other plant material, eggs, animal tissue or organs, marine animals, marine plants or micro-organisms.

The feed material may have been genetically modified.

(2) Particle size of feed material (if a solid feed stream is used).

A small particle size is preferred so that the total surface area is increased to allow the soluble phospholipids to dissolve.

(3) Solvent type.

Methanol, ethanol, n-propanol and iso-propanol and mixtures thereof are all suitable solvents, as are these alcohols with up to 40% v/v water.

However, 95% v/v aqueous ethanol is a preferred solvent as it results in the lowest solubility of PS, CL and GS and the highest solubility of SM.

The inventors have found that using a single solvent system to fractionate the phospholipids and/or gangliosides of interest simplifies fractionation processing. The ability to avoid using multiple solvent systems and/or acids and bases simplifies both the removal of the solvent from the residue and extract fractions, as well as subsequent reuse of the recovered ethanol.

Additionally, the use of a single solvent such as ethanol means that no solvent-lipid reaction products are produced, unlike solvents that are used with acids or bases, or solvents such as acetone. This means the natural state of the residue and extract products are preserved.

(4) Ratio of solvent to feed stream.

This ratio impacts on three main areas: purity, PS, CL and/or GS yield and throughput:

- **Purity:** The higher the solvent ratio, the higher the purity of PS, CL and/or GS achieved in the residue.
- **Yield:** The higher the solvent ratio, the lower the yield of PS, CL and/or GS. All of these compounds have very low but finite solubilities in the solvent, and so an increase in the ratio increases the loss in the extract.
- **Throughput:** The higher the solvent ratio the lower the throughput.

Those skilled in the art will recognise that there are competing constraints in obtaining a high purity product, but also a process that maximises yield and throughput.

(5) Temperature of extraction.

The higher the temperature, the higher the purity of the PS, CL and/or GS in the residue. Increasing the temperature increases the solubility of partially soluble phospholipids in the solvent phase. However, the solubility of the PS, CL and/or GS also increases slightly with temperature which lowers the yield in the residue. The temperature is practically limited by the boiling points of the solvents

As above, there are competing constraints in obtaining a high purity product, but also a process that maximises the yield.

(6) Extraction time

The time that is allowed for the extraction to take place affects throughput and purity of PS in the residue. It is obvious that the longer the process takes, the less batch-wise extractions that can be carried out. However, sufficient time is required to dissolve the soluble phospholipids. This is very important for extraction of streams that are introduced as suspended particles.

As above, there are competing constraints in obtaining a high purity product, but also a process that maximises throughput.

(7) Agitation.

The degree of agitation must be sufficient to keep the particles suspended and aid the extraction by breaking up any aggregates that form. Those skilled in the art will recognise that high shear mixing would be the most advantageous at a large scale.

However, while agitation generally breaks aggregates up, high shear agitation may also bring the particles together in collisions that could form aggregates. This is a concern when less than optimal agitation is employed. For example it is a real risk in scale up where factors like tank diameter and height, mixing speed, impeller type and size come into play.

When the lipid-rich feed material is a solid, it is also possible to carry out the separation process using packed beds with no agitation. The solvent can be passed through the packed bed of ground feed material at a low temperature to extract compounds with the highest solubility (and lowest melting point), and then increasing the temperature (either stagewise or continuously) until the desired final temperature is achieved that gives the highest purity of PS, CL and/or GS in the residue. This process uses temperature rather than the solvent to feed stream ratio to achieve fractionation. At low temperatures (10 – 20°C) PC is selectively dissolved. Subsequent temperature steps can then be completed to remove the more soluble phospholipids (PE, SM and PI) up to a last step of between 50 and 60°C. The resulting packed bed is enriched in PS and/or CL and gangliosides and is a powdery precipitate.

(8) Ratio of water to solvent

At low water content (< 6 %) the relative PS and/or CL purity compared to other phospholipids is high, the recovery of PS and/or CL is good, and the resultant solvent-insoluble fraction containing PS, CL and/or GS is an easy to handle powder. At low water content, SM is also highly soluble in the solvent. As the water content increases PS becomes more soluble in the solvent, whilst, PC and SM solubility decreases, leading to poorer separation and lower yields.

(9) pH

pH does not affect the fractionation over the range 2 - 9, although acidic conditions give a slight enrichment of PS. However, the use of acids complicates the process and introduces additional costs

As it will be appreciated by those skilled in the art, the variables discussed above may be manipulated to optimise the compositions of the soluble product, for example SM levels, as desired.

The term "derived" as used herein means originating from.

The term "comprising" as used in this specification means "consisting at least in part of". When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner.

Percentages unless otherwise indicated are on a w/w solids basis.

The invention consists in the foregoing and also envisages constructions of which the following gives examples only.

EXAMPLES

Example 1: One Pot Stirred Tank Ethanol Extraction of Dairy Feed Streams

These feed streams, all derived from MFGM rich fractions of bovine buttermilk, having different phospholipids and ganglioside compositions were obtained using processes disclosed in PCT international applications PCT/NZ2004/000014 (published as WO WO2004/066744) and PCT/NZ2005/000262 (published as WO 2006/041316).

Feed Stream A: High in lipid content (up to 94% lipid by Modified Roese-Gottlieb), with approximately 32% phospholipids and 2.3% ganglioside. This Feed Stream was produced by near critical dimethyl ether extraction of a MFGM rich fraction of bovine buttermilk with reduced lactose content.

Feed Stream B: Contains high levels of phospholipids, 65% by weight. This feed stream was produced by supercritical CO_2 extraction of the MFGM rich fraction of bovine buttermilk with reduced lactose content (to remove neutral lipids) prior to near critical dimethyl ether extraction.

Feed Stream C: Feed Stream C has a very high lipid content and a proportionally higher amount of phospholipids than Feed Stream A, but almost no gangliosides. This Feed Stream was produced by near-critical dimethyl ether extraction of a MFGM rich fraction of bovine buttermilk with reduced lactose content.

The phospholipids and ganglioside compositions of the three feed streams are outlined in Table 1 below.

Table 1: Phospholipid and ganglioside compositions of Feed Streams A-C

	Feed Stream	A	B	C
	% PL	31.0%	65.6%	41.7%
% of PL	PC	23.7%	23.4%	26.9%
	PI	8.2%	8.1%	6.8%
	PS	12.7%	12.2%	10.4%
	PE	33.1%	32.9%	31.8%
	SM	18.3%	18.8%	18.8%
	DHSM	3.7%	4.2%	
	% PS	3.9%	8.0%	4.3%
	% SM	6.8%	15.1%	7.8%
	GS	2.3%	3.6%	0.6%

Each feed stream was introduced to an excess of solvent (95% ethanol) at a temperature above 50°C and agitated for a determined length of time. During this process the ethanol soluble phospholipids were dissolved into the ethanol producing an extract stream, and leaving behind the insoluble material ("residue") which as a result has enriched levels of PS and GS.

Details of the extractions are provided under Examples 1a – 1d below.

Feed Streams A and C are the easiest to process. They are liquid at 40°C and so can be pumped. They are also both solid at 0°C so can be fed through a Shiver. This provides two options for introducing these feed streams to the solvent, because in either form (liquid or frozen), with ethanol at elevated temperatures, both dissolve readily.

Feed Stream B is a solid gum at all practical processing temperatures so it requires cryogenic milling before ethanol extraction. Thus for this process Feed Stream B needed to be milled to a fine powder (this required cryogenic milling) before introduction into the solvent. This additional processing step adds costs, making it significantly more expensive to process than Feed Streams A or C. There is also a risk of not milling Feed Stream B sufficiently, which would compromise the process and cause a lower achieved purity. However, this problem may be overcome by dispersing the feed stream in a small amount of solvent first.

Feed Streams A and C are liquid at elevated temperatures because of the high neutral lipid content while Feed Stream B is solid at all practical temperatures because of its low neutral lipid content.

Example 1a

1. 100 mL of 95% ethanol was placed in a 100 mL Schott bottle in a water bath at 60°C.
2. The ethanol was agitated by an overhead stirrer (Janke&Kunkel IKA- WERK RW20 overhead stirrer) until the temperature reached 60°C.
3. Approximately 2.5 g of feed stream A was weighed into a 10 ml syringe.
4. The syringe was then heated to 60°C to liquefy the feed stream.
5. The liquefied feed stream A was then added to the ethanol over a 5 minute period by depressing the syringe.
6. The feed stream / ethanol mixture was then stirred for 2 hours at 60°C.
7. The solution was then passed through a Whatman 4 filter paper and 0.235 g of insoluble material (the residue) was collected. The ethanol mixture that passed through the filter contained the remaining 2.265 grams of phospholipid material (the extract).
8. Fractions of the residue and extract were analysed for phospholipids content by ^{31}P-NMR. The residue was found to be highly enriched in PS as shown in table 2 (GS levels were not measured). The extract composition was found to have enriched levels of SM, also as shown in table 2.

Example 1b

1. 100 mL of 95% ethanol was placed in a 100 mL Schott bottle in a jacket heated by a water bath at 60°C.
2. The ethanol was agitated by a magnetic stirrer (Janke&Kunkel IKA Labortechnik RCT basic magnetic stirrer) until its temperature reached 60°C.
3. 2.1744 g of feed stream A was added to a 10 ml syringe.
4. The syringe was then heated to 60°C to liquefy the feed stream.

5. The liquefied feed stream A was then added to the ethanol over a 5 minute period by depressing the syringe.

6. The feed stream / ethanol mixture was then stirred for 2 hours at 60°C.

7. The solution was then passed through a Whatman 4 filter paper and 0.145 g of insoluble material (the residue) was collected. The ethanol mixture that passed through the filter contained the remaining 2.0294 grams of phospholipid material (the extract).

8. The residue fraction was analysed for phospholipids content by ^{31}P-NMR. The residue was found to be highly enriched in PS as shown in table 2 (GS levels were not measured).

Example 1c

This example shows a method for processing solid lipid-rich substrates.

1. 24.6 grams of Feed Stream B was cryogenically milled.

2. The milled material was placed in a 1 L Schott bottle and 200 mL of 95% ethanol at a temperature of < 4 °C was poured into the bottle

3. The bottle was placed into a temperature controlled water bath set at 65 °C. Stirring was carried out as for Example 1b.

4. 800 mL of 95% ethanol at a temperature of 65 °C was then added to the mixture in the bottle, to give a feed to solvent ratio of 1:50.

5. The feed stream / ethanol mixture was continually stirred and the temperature was maintained at 65 °C.

6. After three hours the ethanol mixture was poured through Whatman 4 filter paper and 6.8 grams of insoluble material was collected (the residue) The ethanol mixture that passed through the filter (the extract) contains the remaining 17.3 grams of phospholipid material.

7. Fractions of the residue and extract were analysed for phospholipids content by ^{31}P-NMR. The residue was found to be highly enriched in PS and GS as shown in table 2. The extract composition was found to have enriched levels of SM, also as shown in table 2.

Example 1d

This example shows that PS is substantially enriched when the feed stream is low in GS and lactose.

1. 100 grams of 95% ethanol was placed in a 100 mL beaker on a hot plate with magnetic stirrer, and agitated as for Example 1b whilst simultaneously raising the temperature to 65°C.

2. 2.0 grams of Feed Stream C was added to the ethanol, and the mixture was continuously stirred at 65°C for 2 hours.

3. The mixture was then poured through Whatman 4 filter paper and 0.12 grams of insoluble material was collected (the residue). The ethanol mixture that passed through the filter (the extract) contains the remaining 1.87 grams of phospholipid material.

4. Fractions of the residue and extract were analysed for phospholipids content by ^{31}P-NMR. The residue was found to be highly enriched in PS as shown in table 2. The extract composition was found to have elevated levels of SM, also as shown in table 2.

Table 2 shows the results achieved by this process.

Table 2: Comparison of the results achieved by the processes of Example 1

Example		1a		1b		1c		1d	
Feed Stream		A		A		B		C	
Solvent:Feed		30:1		50:1		50:1		50:1	
Output		Residue	Extract	Residue	Extract	Residue	Extract	Residue	Extract
Yield (solids)		9%	91%	7%	93%	27.6%	72.4%	6%	94%
%PL		62.2	37.5	64.2		51.7	63.7	89.3	41.2
% of PL	PC	2.7	31.0	1.9		3.6	29.6	0.8	29.6
	PI	18.8	4.5	16.1		18.1	5.3	7.8	6.4
	PS	52.7	2.2	60.6		44.5	2.6	82.5	1.2
	PE	22.0	34.1	17.5		27.5	34.3	6.0	32.8
	SM	1.6	22.2	1.6		2.7	22.2	0.0	22.8
	DHSM	0.0	5.1	0.0		0.9	5.1	0.0	0.0
% PS		32.7	0.8	38.9		23.0	1.7	73.7	0.5
% SM + DHSM		1.0	10.2	1.0		1.9	17.4	0.0	9.3
GS						6.0	2.7	Low	Low

Example 1e – Low Temperature Extraction

This example shows that low processing temperatures result in low levels of enrichment of PS.

1. 0.7857 grams of Feed Stream B was cryogenically milled, and placed in a 50 mL centrifuge tube.
2. 47.5 mL of 95% ethanol at a temperature of 37°C was then added to the tube, which was then added to a mixing rack in mechanical shaker water bath set at 37°C.
3. Mixing was then carried out for 2 hours at 37°C, and then the mixture was passed through a Whatman 4 filter.

0.28 grams of insoluble material was collected (the residue) and found to have a PS content of 19.4% (^{31}P-NMR analysis).

Example 2: Multi Step Stirred Tank Ethanol Extraction of Dairy Feed Stream

This example shows that a multistep stirred tank extraction process with lower solvent to feed ratios than those used in Example 1 can also achieve significant enrichment of PS and GS.

Feed Stream B of Example 1 was also used in this example. As for Example 1, Feed stream B was cryogenically milled to a fine powder before introduction into the solvent.

The process was carried out as follows:

1. 500 grams of cryogenically milled feed stream B was placed in a 5 L vessel, and 5L of 95% ethanol at 65°C was added.
2. The vessel was shaken periodically and the temperature was maintained at 65 °C by placing the vessel in a hot water bath.
3. After 1 hour, the shaking was stopped and the insoluble material allowed to drop to the bottom of the vessel.
4. The solvent phase was decanted, and put aside.

5. The solid material from this first extraction was recovered, frozen, and remilled cryogenically.

6. The remilled material was placed in a 5L vessel, and 5L of 95% ethanol at 65°C was added. The vessel was then shaken periodically and the temperature was maintained at 65°C by placing the vessel in a hot water bath.

7. After one hour, the solution was filtered through Whatman 4 filter paper and the powder residue was collected. The solvent phase was recovered and combined with the solvent phase from the first extraction.

8. The powder residue was placed in a 5L vessel and 5L of 95% ethanol at 65°C was added. The vessel was shaken periodically and the temperature was maintained at 65°C by placing the vessel in a hot water bath.

9. After two hours and 30 minutes the mixture was filtered through Whatman 4 filter paper. The recovered solids were freeze dried, giving 125.3 g of powder ("residue"). The filtrate was collected, and evaporated ("Extract 4")

10. The combined solvent phases from step 7 were held at 20°C for four days, during which further precipitation of phospholipids occurred. The solution was then filtered through Whatman 4 filter paper. The powder residue was collected and freeze dried (78.8 g of powder – "Extract 1"). The filtrate was collected and stored in the freezer at <0°C for 24 hours which caused further precipitation, after which it was filtered through Whatman 4 filter paper. The filtrate was then collected and evaporated and freeze dried, and 110.6 grams of powder was produced ("Extract 2"). The filter solids were freeze dried producing 85.0g of powder ("Extract 3").

11. The residue and Extracts 1-4 were analysed for phospholipids content by ^{31}P-NMR. The residue was found to be highly enriched in PS and GS. The compositions are shown in table 3.

Table 3 shows the results achieved by this process.

Table 3: Residue and extract fraction compositions from multistep extraction

Feed Stream		B				
Output		Residue	Extract 1	Extract 2	Extract 3	Extract 4
Yield (solids)		25%	16%	22%	17%	6%
%PL		52	47	70	73	63
% of PL	PC	1	11	53	24	12
	PI	23	10	0	1	10
	PS	46	6	0	1	3
	PE	28	36	28	31	56
	SM	0	37	18	43	17
% PS		24	3	0	1	2
% SM		0	17	12	31	11
GS		6.0	4.2	0.0	0.4	

Example 3: Packed Bed Ethanol Extraction of Solid Feed Streams

This example shows that solid mixtures of phospholipids can also be separated using a packed bed method with no agitation. Dairy feed stream B was used, but the general method is applicable to soy lecithin, and phospholipids concentrates from animal organs and tissues. The feed stream is cryogenically milled to a fine powder.

This process uses temperature rather than the solvent to feed stream ratio to achieve fractionation.

This process was developed to overcome the "gumming up" and aggregation problems which were observed when using feed stream B (or other feed streams that contain very low levels of neutral lipids) in processes such as those described in Examples 1 and 2.

The current belief is that the "gumming up" is caused by the phosphatidyl choline (PC) not being solublised sufficiently and forming a gel. This packed bed process mitigates this issue by using a low temperature step (10 – 20°C).

In this temperature range the PC is selectively dissolved and because there are no competing phospholipids being dissolved, the PC is removed from the packed bed without forming a gel.

Increasing temperature steps can then be completed to remove the other phospholipids (PE, SM and PI) up to a last step of between 50 and 60°C.

The resulting packed bed is enriched in PS and gangliosides and is a powdery precipitate

This process is also advantageous as at temperatures above 60°C PS becomes soluble and significant losses can be experienced.

Another advantage of this technique is the elimination of the need for high shear agitation. While agitation generally breaks aggregates up, high shear agitation may also bring the particles together in collisions that could form aggregates. This is a concern when less than optimal agitation is employed. For example it is a real risk in scale up where factors like tank diameter and height, mixing speed, impeller type and size come into play.

Further, this process uses non mixing (laminar) gentle fluid dynamics to contact the feed and the ethanol, so there is minimal collision and hence less chance for aggregates to form.

The process was carried out in the following manner:

1. A known amount of milled Feed Stream B (5.1062 grams) was spread evenly inside a jacketed sinter filter.
2. The sinter jacket was connected to a temperature controlled water bath with a pump.
3. The water bath was set to 10°C.
4. 100mL of 95% ethanol (volume equals 20 times the amount of Feed Stream B, e.g. 20 mL of ethanol per 1 gram of Feed Stream B) was measured into a vessel and placed in the water bath.
5. Once the ethanol and bed reached 10°C the ethanol was poured into the top of the sinter and flowed through the bed into a flask.
6. The ethanol was recycled and continually poured through the bed for 24 minutes. The material in the bed which was soluble at 10°C was dissolved into the ethanol.
7. The ethanol and first phospholipid extract were then collected and the ethanol evaporated, yielding 0.613 grams of a phospholipid mixture described in Table 4 as 10°C Extract;

8. The water bath temperature was raised to T2, 20°C.

9. 100mL of fresh 95% ethanol was measured out and placed in the water bath.

10. Once the ethanol and bed reached T2 20°C, the ethanol was poured into the top of the sinter and flowed through the bed into a flask.

11. The ethanol was recycled and continually poured through the bed for 28 minutes. The material in the bed which was soluble at 20°C was dissolved into the ethanol.

12. The ethanol and second phospholipid extract were then collected, yielding 1.021 grams of a phospholipid mixture described in Table 4 as 20°C Extract

13. The water bath temperature was raised to 30°C.

14. 100mL of fresh 95% ethanol was measured out and placed in the water bath.

15. Once the ethanol and bed reached 30°C the ethanol was poured into the top of the sinter and flowed through the bed into a flask.

16. The ethanol was recycled and continually poured through the bed for 30 minutes. The material in the bed which was soluble at 30°C was dissolved into the ethanol.

17. The ethanol and third phospholipid extract were then collected, yielding 0.970 grams of a phospholipid mixture described in Table 4 as 30°C Extract.

18. The water bath temperature was raised to 50°C.

19. 100mL of fresh 95% ethanol was measured out and placed in the water bath.

20. Once the ethanol and bed reached 50°C the ethanol was poured into the top of the sinter and flowed through the bed into a flask.

21. The ethanol was recycled and continually poured through the bed for 53 minutes. The material in the bed which was soluble at 50°C was dissolved into the ethanol

22. The ethanol and fourth phospholipid extract were then collected, yielding 0.521 grams of a phospholipid mixture described in Table 4 as 50°C Extract

23. The water bath temperature was raised 60°C.

24. 100mL of fresh 95% ethanol was measured out and placed in the water bath.

25. Once the ethanol and bed reached 60°C the ethanol was poured into the top of the sinter and flowed through the bed into a flask.

26. The ethanol was recycled and continually poured through the bed for 22 minutes. The material in the bed which was soluble at 60°C was dissolved into the ethanol

27. The ethanol and fifth phospholipid extract were then collected, yielding 0.117 grams of a phospholipid mixture described in Table 4 as 60°C Extract

28. The bed residue was collected, 1.277 grams, this was found to contain elevated levels of PS and gangliosides. This is described in Table 4 as the residue.

Table 4 shows the results achieved by this process.

Table 4: Results achieved by the process of Example 3.

Feed Stream		**B**					
Output		**Residue**	**10°C Extract**	**20°C Extract**	**30°C Extract**	**50°C Extract**	**60°C Extract**
Yield (solids)		**25%**	**12%**	**20%**	**19%**	**10.5%**	**2.3%**
%PL		64.1	66.8	82.2	72.3	73.0	64.4
%	**PC**	1.4	44.8	39.2	26.9	12.3	4.6
of	**PI**	22.4	1.1	0.7	3.5	11.1	24.1
PL	**PS**	48.8	0.8	0.5	2.5	4.7	17.1
	PE	23.5	35.5	26.1	28.2	48.2	43.4
	SM	1.2	14.5	26.7	30.3	17.7	8.1
	DHSM	0.0	2.3	5.8	8.1	4.5	1.9
% PS		31.2	0.5	0.4	1.8	3.5	11.0
% SM		0.8	11.2	26.7	27.8	16.2	6.4

The extracts from each step can be combined or left as separate phospholipid fractions. For example the 20°C and 30°C extracts are at least 25% SM product, while others could be used as a PC/PE phospholipid mixture.

Discussion of Examples 1-3

The processes used in Examples 1-3 were laboratory scale. In assessing whether the processes would be scaleable to commercial plants, the inventors considered the following factors.

Feed Stream (feed material)

Three different feed streams were used in Example 1.

Feed streams A and C proved easiest to use, as they are liquid at temperatures above 40°C and hence are dispersed into the solvent with ease.

Feed Stream B on the other hand is a gummy solid at temperatures above 0°C. Below 0°C the solid becomes rigid and cryogenic milling can be used to reduce the feed stream to fine powder. This allows sufficient dispersion in the extraction solvent. The need for cryogenic milling to disperse Feed Stream B makes it the least preferred feed stream of the three streams that were trialled, although as noted this problem could be overcome by dispersing the feed stream in a small amount of solvent first.

As discussed above, the current belief is that the "gumming up" is caused by the phosphatidyl choline (PC) not being solublised sufficiently and forming a gel.

As between Feed Streams A and C, Feed Stream A is preferred because it contains a much higher proportion of gangliosides which means that the residue (produced using 30:1 solvent to feed stream ratio) contains both a reasonable level of PS (32.7% PS) and a ganglioside content of around 6%. Thus even though the residue produced using Feed Stream C contained a higher level of phospholipids, it is desirable for many health applications to produce a product enriched in both PS and gangliosides making Feed Stream A the preferred stream of the two.

Further, the co-product ("extract") from Feed Stream A (produced using 30:1 solvent to feed stream ratio) contained 10.2% SM. However this product was also high in neutral lipids and contained only 37.5% phospholipids. The product could be processed further to remove the neutral lipids using supercritical CO_2, degumming or acetone.

As already discussed, the residue produced by extraction of Feed Stream C produced the highest enrichment of PS (73.7%), but the ganglioside content of this product is lower than that of the residues produced by Feed Streams A and B. Feed stream C contains fewer

impurities than Feed Stream A. This highlights the fact that reducing the lactose and ash content of the feed stream upfront will have a very positive effect on the purity of the PS extract that can be attained.

Finally, although Feed Stream B was the least desirable stream, because of its gummy nature, the residue was similar to the residue from extraction using Feed Stream A, having similar levels of PS (23.0%) and gangliosides (6.0%). And the co-product ("extract") is more attractive, because it contains very low levels of neutral lipids, high levels of phospholipids (63.7%) and has 14.1% SM.

While Feed Stream A is the preferred stream for the process described in Example 1, the yield is of concern, as only 9% of the total feed stream solids is converted to a PS enriched product. This meant the throughput of the process is low, and 91% of the feed stream solids ended up as the co-product (the "extract").

However the yield of the extraction of Feed Stream C is even lower: 6% of the feed stream solids was converted to PS. However the purity of the PS product is very high.

Products of Multi Step process (Example 2)

Example 2 differs from Example 1 in that many co-products ("extracts") were produced which could be kept separately.

The extraction can produce up to four co-products, as summarised in Table 3, the most notable being the 31% SM product (Extract 3).

Packed Bed Extraction (Example 3)

Probably the most attractive aspect of this technique is the fractionation that occurs during the extraction. It is obvious that with some manipulation of the ethanol temperatures in the steps an enriched SM product can also be produced.

The process achieved complete fractionation of Feed Stream B into fractions containing high sphingomyelin and phosphatidyl serine.

However, one drawback of this option is the difficult in handling Feed Stream B, as discussed.

Solvent : Feed Stream Ratio

It was also discovered that the solvent to feed ratio directly affects the PS concentration in the insoluble fraction. A general rule is: the higher the solvent ratio the higher the purity of the PS in the insoluble fraction.

For example, it was found in Example 1 that the solvent ratio of 30:1 with Feed Stream A yields a 32.7% PS fraction. With a 50:1 solvent to feed ratio the fraction is 38.9% PS.

The 30:1 solvent ratio also has advantages however. This lower solvent ratio allows for smaller extraction vessels at full scale so will minimise cost and improve throughput.

Temperature & Time

Both time and temperature also affected the PS purity in the residues.

These variables both have obvious practical limits for a full-scale operation. The recommendation is to have the temperature as high as is economically and practically possible for the length of time needed to achieve a reasonable level of PS in the final product.

Here the inventors are interested in a product containing at least 30% PS. In this study, both Feed Streams A and C in the process of Example 1 yielded a product that exceeds the 30% PS target.

Example 4: Effect of water content on the ethanol fractionation of Dairy Lipid extract (Feed Stream B)

This example shows the effect of water content on PS enrichment at 65°C.

0.8 g of dairy lipid extract, Feed Stream B, was mixed in 40ml of 95% ethanol (0% to 40% water). The mixture was then rapidly heated to 65°C and shaken vigorously. Samples were kept at 65°C for 3 hours with periodic shaking. The samples were then filtered and the solids air dried followed by drying under vacuum. The samples were analysed by ^{31}P NMR. The results are shown in Table 5.

At lower water content the relative PS purity compared to other phospholipids is high, the recovery of PS is good, and the fraction is an easy to handle powder. As the water content increases the recovery of PS decreases, PC and SM content increases in the product, and fractions containing more water and hydrated phospholipids are produced.

Table 5

Water content (%)	Yield (% solids)	% GS	% PL	Phospholipid composition (%)				
				PC	PI	PS	PE	SM
Feed		3.6	65.6	23.4	8.1	12.2	32.9	23.0
0	33.6	4.1	52.3	3.8	20.6	38.5	32.8	2.7
5	28.0	4.2	50.6	3.2	20.0	43.0	29.5	2.5
10	20.7	4.6	51.4	4.7	17.4	38.0	32.9	5.5
20	18.9	4.0	62.7	5.1	14.6	44.8	27.8	6.0
30	35.0	3.9	67.8	11.7	11.7	22.6	36.0	17.9
40	20.0	3.5	78.5	19.1	9.4	13.5	35.8	21.8

Example 5: Effect of sample to solvent ratio on ethanol fractionation of Dairy Lipid extract (Feed Stream B)

This example shows the effect of sample to solvent ratio on PS enrichment at 65°C.

2g of dairy lipid extract, Feed Stream B, was added to 20ml 95% ethanol and mixed at 65°C. Cold ethanol was added to achieve the desired ratio of sample to solvent followed by mixing. After 1 hour in the freezer the samples were mixed again, then heated to 65°C and re-mixed. The samples were left overnight at 65°C, then mixed, and filtered. All mixing was carried out using an ultraturrex. The collected solids were dried under vacuum and analysed by ^{31}P NMR. The results are shown in Table 6 below. PS enrichment was improved by increasing the amount of solvent. At a ratio of 1:100 sample:solvent, 74.3% of the phospholipids in the insoluble material was PS. With phospholipids making up 56.6% of this fraction, the amount of PS in this sample was 42%.

Table 6

Sample:solvent	Yield (% solids)	% PL	Phospholipid composition (%)				
			PC	PI	PS	PE	SM
Feed			23.9	7.9	11.3	33.1	22.2
1:10	35	56.9	9.8	15.3	24.2	35.9	11.6
1:20	21	55.7	3.6	20.9	35.9	32.0	4.7
1:50	17	56.1	1.8	12.7	67.0	13.5	1.5
1:100	15.4	56.6	1.2	10.2	74.3	8.7	1.0

Example 6: Effect of pH on ethanol fractionation of Dairy Lipid extract (Feed Stream B)

This example shows the effect of pH on PS enrichment at 65°C.

Dairy lipid extract (0.8g) (Feed Stream B) was added to 40ml of 95% ethanol at 65°C. The ethanol contained either 5% water, 5% glacial acetic acid, or 5% aqueous ammonia (2.5%), and the pH of the three mixtures were 7.7, 3.2 and 10.0 respectively. The mixtures were agitated by periodic shaking over 2 hours, then filtered. The fractions were dried under vacuum and the analysed by ^{31}P NMR. The results are shown in Table 7 below. Acidic conditions resulted in the best PS enrichment, but the results were similar across the wide pH range.

Table 7

	Yield (% solids)	% PL	Phospholipid composition (%)				
			PC	PI	PS	PE	SM
Feed			23.9	7.9	11.3	33.1	22.2
pH = 3.2 solid	24	59.7	3.5	23.3	44.5	23.8	3.0
pH = 7.7 solid	31	46.8	5.5	16.3	42.1	28.9	5.0
pH = 10.0 solid	33	52.0	4.8	17.7	39.3	31.4	5.0

Example 7: Effect of solvent on fractionation of Dairy Lipid extract (Feed Stream C)

This example shows the effect of the alcohol chain length on PS enrichment.

100g of solvent, with 5% water, was heated to 65°C (50°C for methanol due to a lower boiling point) on a stirred hotplate. Once at temperature, 2g of dairy lipid extract (Feed Stream C) was added and mixed for two hours. The sample was removed from the heat and filtered under vacuum while still hot. The insoluble fraction was then dried on Whatman 4 filter paper and the soluble fraction was rotary evaporated. All weights were recorded to carry out a mass balance. Samples were analysed by ^{31}P NMR. The results are shown in Table 8.

Table 8

	Yield (% solids)	% PL	Phospholipid composition (%)				
			PC	PI	PS	PE	SM
Feed		41.7	26.9	6.8	10.4	31.8	22.9
Methanol							
Insoluble	42	13.1	0.9	11.2	64.7	21.7	1.3
Soluble	53	56.6	31.3	6.4	1.4	32.1	27.5
Ethanol							
Insoluble	7	89.3	0.8	7.8	82.5	6.0	N/D
Soluble	93	41.2	29.6	6.4	1.2	32.8	28.0
n-Propanol							
Insoluble	3	75.2	0.5	6.6	88.0	2.6	N/D
Soluble	96	36.8	31.5	4.3	3.6	33.6	26.2
iso-Propanol							
Insoluble	6	86.6	0.7	11.0	80.5	4.6	0.6
Soluble	93						

Example 8: Ethanol Fractionation of Phospholipids from various sources (sample to solvent 1:10)

This example shows that the process of the invention is broadly applicable to a wide range of lipid substrates using a sample to solvent ratio of 1:10 and a temperature of 50°C. This example also shows that cardiolipin is substantially concentrated by a process of the invention when present in the feed material.

Phospholipid rich extract (0.5g) from the following sources:

- bovine liver,
- bovine heart,
- shark spinal cord,
- lemon fish, and
- hoki head

were weighed in test tubes and 5g of 95% ethanol added. The test tube was stoppered and placed in a water bath at 50°C, with periodic shaking and ultrasonication over a 2 hour period. The test tubes were removed and centrifuged (3000 rpm, 2min) and the supernatant decanted from the solid. Solvent was removed from the fractions under a stream of argon and then under vacuum. Phospholipid profiles were determined by ^{31}P NMR. The results are shown in Table 9.

In all cases the desired phospholipids PS and CL were enriched in the insoluble fraction. The phospholipids, especially those from lemonfish, have higher levels of polyunsaturated fatty acids than dairy lipids, which increase their solubility in ethanol. SM was enriched in the soluble fraction.

Table 9

	Yield (% solids)	% PL	Phospholipid composition (%)					
			PC	PI	PS	PE	SM	CL
Bovine Liver								
Feed		56.7	44.9	10.4	2.2	23.0	3.6	8.3
Insoluble	12	73.0	9.1	29.1	6.2	33.8	N/D	16.0
Soluble	88	43.2	56.9	3.8	0.9	20.7	4.3	6.1
Bovine Heart								
Feed		61.1	21.7	4.4	2.0	20.8	5.4	26.4
Insoluble	27	72.9	4.4	14.0	5.1	21.6	6.6	41.1
Soluble	73	55.4	34.9	1.0	0.5	25.5	6.6	15.8
Shark spine								
Feed		22.9	35.7	1.4	12.6	47.5	2.8	N/D
Insoluble	22	43.5	21.3	2.7	23.4	46.8	3.9	N/D
Lemonfish								
Feed		63.7	46.2	7.9	3.5	25.0	5.2	7.9
Insoluble	26	76.1	8.3	27.6	10.7	29.4	N/D	19.6
Soluble	74	50.5	58.7	2.0	1.3	24.6	5.8	4.2
Hoki head								
Feed		33.2	49.6	5.3	6.3	22.8		3.1
Insoluble	23	26.1	25.6	10.2	13.4	22.1		10.6
Soluble	77	36.2	52.2	4.5	5.4	24.5		2.9

The Hoki head PE and SM concentrations have been grouped together because of poor resolution during analysis.

Example 9: Ethanol Fractionation of Phospholipids from various sources (sample to solvent 1:50)

This example shows that PS can be enriched from feed materials in which the level of PS is low.

100g of 95% ethanol was heated to 65°C on a stirred hotplate. Once at temperature, 2g of sample was added and mixed for two hours. The sample was removed from the heat and filtered under vacuum while still hot. The insoluble fraction was caught then dried on Whatman 4 filter paper and the soluble fraction was rotary evaporated down. All weights were recorded to carry out a mass balance. Samples were analysed by ^{31}P NMR. The results are shown in Table 10.

Table 10

	Yield (% solids)	% PL	Phospholipid composition (%)					
			PC	PI	PS	PE	SM	CL
Soy Lecithin Feed		66.3	33.5	19.8	N/D	29.1	N/D	N/D
Insoluble	24	69.0	6.4	39.4	1.1	29.1	N/D	N/D
Soluble	76	73.2	53.0	7.7	N/D	21.8	N/D	N/D
Egg Feed		27.7	70.2	1.8	1.6	18.8	4.7	N/D
Insoluble	4	29.1	8.4	14.7	48.4	18.0	N/D	N/D
Soluble	96	30.3	71.0	1.8	N/D	18.0	4.4	N/D

INDUSTRIAL APPLICATION

The present invention has utility in providing products with high levels of particular phospholipids/sphingolipids including phosphatidyl serine, gangliosides and sphingomyelin. These products may be employed in a number of applications, including infant formulas, brain health, sports nutrition, drug delivery, dermatological applications, and arthritis.

REFERENCES

Jensen, R.G. 2002. The Composition of Bovine Milk Lipids: January 1996 to December 2000. J. Dairy Sci. 85: 295-350

Ladisch, S.; Gillard, B. 1985. A solvent partition method for microscale ganglioside purification. Anal. Biochem. 146: 220-231

Padley F.B.; Gunstone, F.D.; Harwood, J.L. 1986. Occurrence and characteristics of oils and fats, in The Lipid Handbook (eds. Gunstone, F.D.; Harwood, J.L.; Padley, F.B.) pp. 49-170. Chapman and Hall, London – NY.

Wu, Y.; Wang, T. 2004. Fractionation of crude soybean lecithin with aqueous ethanol. JAOCS 81(7): 697-704

WHAT WE CLAIM IS:

1. A process for fractionating a feed material into soluble and insoluble components, comprising

(a) providing a feed material comprising one or more of:

(i) at least 1% w/w phosphatidyl serine,

(ii) at least 1% w/w sphingomyelin,

(iii) at least 1% w/w cardiolipin, or

(iv) at least 0.3% w/w gangliosides

(b) providing a solvent comprising one or more C_1-C_3 monohydric alcohols and water, wherein the water content of the one or more alcohols is 0 to 40% v/v

(c) contacting the feed material and the solvent and subsequently separating the solvent containing the soluble components from the insoluble components

(d) optionally separating the soluble components and the solvent.

2. The process of claim 1 wherein the feed material comprises less than 10% protein, and less than 10% lactose.

3. The process of claim 1 wherein less than 10% of the feed material comprises protein and lactose.

4. The process of claim 1 wherein the feed material comprises greater than 1% phosphatidyl serine.

5. The process of claim 1 wherein the feed material comprises greater than 2% phosphatidyl serine.

6. The process of claim 1 wherein the feed material comprises greater than 5% phosphatidyl serine.

7. The process of claim 1 wherein the feed material comprises greater than 1% sphingomyelin.

8. The process of claim 1 wherein the feed material comprises greater than 5% sphingomyelin.

9. The process of claim 1 wherein the feed material comprises greater than 15% sphingomyelin.

10. The process of claim 1 wherein the feed material comprises greater than 1% cardiolipin.

11. The process of claim 1 wherein the feed material comprises greater than 2% cardiolipin.

12. The process of claim 1 wherein the feed material comprises greater than 5% cardiolipin.

13. The process of claim 1 wherein the feed material comprises greater than 0.3% gangliosides.

14. The process of claim 1 wherein the feed material comprises greater than 1% gangliosides.

15. The process of claim 1 wherein the feed material comprises greater than 2% gangliosides.

16. The process of any one of claim 1 to 15 wherein the feed material is derived from terrestrial animals, marine animals, terrestrial plants, marine plants, or micro-organisms such as microalgae, yeast and bacteria.

17. The process of claim 16 wherein the feed material is derived from sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human.

18. The process of claim 16 or claim 17 wherein the feed material is selected from: tissue, a tissue fraction, organ, an organ fraction, milk, a milk fraction, colostrum, a colostrum fraction, blood and a blood fraction.

19. The process of claim 16 wherein the feed material is derived from dairy material, soy material, eggs, animal tissue, animal organ or animal blood.

20. The process of claim 16 wherein the feed material is selected from: a composition comprising dairy lipids, a composition comprising egg lipids, and a composition comprising marine lipids.

21. The process of any one of claims 1 to 20 wherein the feed material is a bovine milk fraction.

22. The process of claim 21 wherein the feed material is selected from: buttermilk, a buttermilk fraction, beta serum, a beta serum fraction, butter serum, a butter serum fraction, whey, a whey fraction, colostrum, and a colostrum fraction.

23. The process of any one of claims 1 to 22 wherein the feed material comprises milk fat globule membrane.

24. The process of any one of claims 1 to 23 wherein the feed material has been genetically modified.

25. The process of any one of claims 1 to 24 wherein the feed material is in solid form.

26. The process of claim 25 wherein the feed material is cryomilled before contact with the solvent.

27. The process of any one of claims 1 to 26 wherein the feed material is cryomilled to achieve a mean particle size of 0.1-5mm before contact with the solvent.

28. The process of any one of claims 1 to 27 wherein the solvent comprises:

(a) an alcohol selected from: methanol, ethanol, n-propanol, isopropanol and mixtures thereof; and

(b) 0 – 40% v/v water

29. The process of claim 28 wherein the solvent comprises between 0 and 20% v/v water.

30. The process of claims 28 wherein the solvent comprises between 1 and 10% v/v water.

31. The process of any one of claims 28 to 30 wherein the alcohol is ethanol.

32. The process of any one of claims 1 to 31 wherein the solvent comprises 95% aqueous ethanol.

33. The process of any one of claims 1 to 32 wherein the ratio of solvent to feed material is between 10:1 to 100:1.

34. The process of claim 33 wherein the ratio of solvent to feed material is between 20:1 to 60:1.

35. The process of claim 33 wherein the ratio of solvent to feed material is between 25:1 to 40:1.

36. The process of any one of claims 1 to 35 wherein the process is carried out at greater than 30°C.

37. The process of claim 36 wherein the process is carried out at greater than 50°C.

38. The process of claim 36 wherein the process is carried out at greater than 55°C.

39. The process of any one of claims 1 to 38 wherein the process is carried out in a number of steps of decreasing or increasing temperatures.

40. The process of any one of claims 1 to 35 wherein the process is carried out at less than or equal to 4°C.

41. The process of claim 40 wherein the process is carried out at less than or equal to 0°C.

42. The process of any one of claims 1 to 41 wherein step (c) is carried out for between 1-3 hours.

43. The process of claim 42 wherein step (c) is carried out for 2 hours.

44. The process of any one of claims 1 to 43 wherein the solvent and feed material are contacted once.

45. The process of any one of claims 1 to 43 wherein the solvent and feed material are contacted multiple times with the same batch of solvent.

46. The process of any one of claims 1 to 43 wherein the feed material is contacted with more than one batch of solvent.

47. The process of any one of claims 1 to 46 wherein the solvent and feed material are contacted by using a packed bed of feed material with the solvent flowing through it.

48. The process of any one of claims 1 to 46 wherein the solvent and feed material are mixed by agitation.

49. The process of claim 48 wherein the agitation is achieved by high shear mixing.

50. A product produced by the process of any one of claims 1 to 49.

51. The product of claim 50 wherein the product is the soluble material.

52. The product of claim 50 wherein the product is the insoluble material.

53. The product of claim 50 or claim 51 wherein the product contains more sphingomyelin than the feed material.

54. The product of claim 50 or claim 51 wherein the product comprises greater than 3% sphingomyelin.

55. The product of claim 50 or claim 51 wherein the product comprises greater than 10% sphingomyelin.

56. The product of claim 50 or claim 51 wherein the product comprises greater than 15% sphingomyelin.

57. The product of claim 50 or claim 52 wherein the product contains more phosphatidyl serine than the feed material

58. The product of claim 50 or claim 52 wherein the product comprises greater than 5% phosphatidyl serine.

59. The product of claim 50 or claim 52 wherein the product comprises greater than 30% phosphatidyl serine.

60. The product of claim 50 or claim 52 wherein the product comprises greater than 70% phosphatidyl serine.

61. The product of claim 50 or claim 52 wherein the product contains more gangliosides than the feed material

62. The product of claim 50 or claim 52 wherein the product comprises greater than 2% gangliosides.

63. The product of claim 50 or claim 52 wherein the product comprises greater than 4% gangliosides.

64. The product of claim 50 or claim 52 wherein the product comprises greater than 6% gangliosides.

65. The product of claim 50 or claim 52 wherein the product contains more cardiolipin than the feed material

66. The product of claim 50 or claim 52 wherein the product comprises greater than 5% cardiolipin.

67. The product of claim 50 or claim 52 wherein the product comprises greater than 10% cardiolipin.

68. The product of claim 50 or claim 52 wherein the product comprises greater than 25% cardiolipin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2007/000088

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C11B 7/00 (2006.01) ***C11B 1/10*** (2006.01) ***A23L 1/48*** (2006.01)

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)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI, JAPIO, FSTA, ESPACE, USPTO (phosphatidyl?serine, sphingomyelin, ethanol, methanol)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/0170063 A1 (CHORDIA et al.) 4 August 2005 See Example II.	1,28-32,50-51
X	WO 2004/047554 A1 (PHARES PHARMACEUTICAL RESEARCH NV) 10 June 2004 See Example 2.	1,28-32,50-51
X	US 5,532,141 A (HOLLER) 2 July 1996 See entire document.	1,28-32,50-51
A	Patent Abstracts of Japan, JP 2002-241385 A (YAKULT HONSHA CO LTD) 28 August 2002 See abstract.	1, 50

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
25 June 2007

Date of mailing of the international search report
28 JUN 2007

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer
ALBERT S. J. YONG
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No : (02) 6283 2160

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/NZ2007/000088

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	2005170063						
WO	2004047554	AU	2003283429	CA	2507243	EP	1569525
		IS	7861	NO	20052498	US	2006128665
US	5532141						
JP	2002241385						

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX