DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

DOCKETS TRANSMITTAL MEMO

0413 '03 JAN 27 P2:03

Date: JAN 23 2003

From: Consumer Safety Officer, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-821

Subject: 75-Day Premarket Notification of New Dietary Ingredients

To: Dockets Management Branch, HFA-305

Subject of the Notification: Neptune Krill Oil™ (Krill Oil Extract)

Firm: Neptune Technologies & Bioressources

Date Received by FDA: 5-15-02

90-Day Date: 8-13-02

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned substance should be placed on public display in docket number 95S-0316 as soon possible since it is past the 90-day date. Thank you for your assistance.

Gloria Chang
Gloria Chang, R.Ph./Interdisciplinary Scientist

Attachments

95S-0316

RPT 131

DEPARTMENT OF HEALTH & HUMAN SERVICES — Public Health Service

Food and Drug Administration
Washington, DC 20204

OCT 4 2002

Tina Sampalis, M.D., Ph.D
Vice President of Research
Neptune Technologies & Bioressources Inc.
500 St. Martin Blvd. West
Laval, Quebec
H7W 3J8, Canada

Dear Dr. Sampalis:

This is to inform you that your notification, dated February 17, 2002 was originally filed with the Food and Drug Administration (FDA) on February 28, 2002. Your original notification contained three separate notifications concerning three new dietary ingredients that were identified as: Neptune Krill Oil™ (Krill oil), Neptune Aquateine™ (Krill-based protein concentrate powder of the dry residue from the extraction of oil), and Neptune LyO-Krill™ (freeze-dried Krill). You indicated that the primary source of all three ingredients is Krill, a shrimp-like crustacean identified as Antarctic Krill (species known as *Euphausia superba*). In a telephone conversation with you on April 8, 2002, we informed you that your notification was incomplete in that it did not supply all of the information as required in Title 21 of the Code of Federal Regulations (21 CFR) Part 190.6.

Your amended notification, dated April 26, 2002, containing the additional information was received by FDA on May 15, 2002, which was the revised filing date for the three ingredients cited above. We note that in the amended notification you included a new notification for an ingredient described as Neptune Krill Enzyme™. Subsequently, in telephone conversations with you on June 17 and July 15, 2002, we requested additional information on this ingredient and indicated that the new filing date for Neptune Krill Enzyme™ would be the date that we receive this information. The new filing date for the notification on Neptune Krill Enzyme™ was July 19, 2002. Further, on July 18, 2002 you notified us via electronic mail that you changed the tradename from Neptune Krill Enzyme™ to Neptune Krill Euphausia™. Hence, we will be using the new tradename for the rest of this letter. FDA considered the notification for Neptune Krill Euphausia™ separately from the other three notifications. Nonetheless, this letter addresses the four notifications for the following ingredients: Neptune Krill Oil™ (Krill oil), Neptune Aquateine™ (Krill-based protein concentrate powder of the dry residue from the extraction of oil), Neptune LyO-Krill™ (freeze-dried Krill), and Neptune Krill Euphausia™) (a blend of Neptune Krill Aquateine™ and Neptune Lyo-Krill™).

In your amended notification, you indicated that the serving levels and daily servings are: Neptune Krill Oil™ (1 gram of oil per gelcap with a recommended daily serving of 1 to 3 gelcaps per day), Neptune Aquateine™ (300 mg per sac with a recommended daily serving of 1 to 3 sacs per day), Neptune LyO-Krill™ (300 mg per capsule with a recommended daily

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serving of 1 to 3 capsules per day) and Neptune Krill Euphausia™ (300 mg per capsule with a recommended daily serving of 1 to 3 capsules. You also stated that there was no limitation in the duration of use for any of the four ingredients and that the only subpopulations excluded from using these ingredients are persons with seafood allergies and those taking anticoagulants.

In accordance with 21 C.F.R 190.6 (c), FDA must acknowledge its receipt of a notification for a new dietary ingredient. For 75 days after the filing dates, you must not introduce or deliver for introduction into interstate commerce any dietary supplement that contains Neptune Krill Oil™ (Krill oil), Neptune Aquateine™ (Krill-based protein concentrate powder of the dry residue from the extraction of oil), Neptune LyO-Krill™ (freeze-dried Krill) or Neptune Krill Euphausia™ (a blend of Neptune Krill Aquateine™ and Neptune Lyo-Krill™).

Please note that acceptance of this notification for filing is a procedural matter and, thus, does not constitute a finding by FDA that the new dietary ingredient or supplement that contains the new dietary ingredient is safe or is not adulterated under 21 U.S.C. 342. Importantly, new dietary ingredients for use in dietary supplements that FDA has reviewed through the premarket notification process are not "approved" or "authorized" by the agency.

Although we are not finding at this time that the basis on which you concluded that a dietary supplement containing either Neptune Krill Oil™, Neptune Aquateine™, Neptune LyO-Krill™ and Neptune Krill Euphausia™ will reasonably be expected to be safe is inadequate, FDA is not precluded from taking action in the future against a dietary supplement containing any of these ingredients if it is found to be adulterated or misbranded. It is the manufacturer's or distributor's responsibility to ensure that any dietary ingredient or a dietary supplement marketed in the United States is safe and complies with all applicable requirements of the Federal Food, Drug and Cosmetic Act and implementing regulations in Title 21 of the Code of Federal Regulations as well as any other applicable Federal laws and regulations.

Your notifications for Neptune Krill Oil™ (Krill oil), Neptune Aquateine™ (Krill-based protein concentrate powder of the dry residue from the extraction of oil), Neptune LyO-Krill™ (freeze-dried Krill) and Neptune Krill Euphausia™ (a blend of Neptune Krill Aquateine™ and Neptune Lyo-Krill™) will be kept confidential for 90 days from the date of their receipt. Therefore, your notifications for Neptune Krill Oil™, Neptune Aquateine™, and Neptune LyO-Krill™ and your notification for Neptune Krill Euphausia™ will be placed on public display at FDA's Dockets Management Branch in docket number 95S-0316 after August 13, 2002 and October 16, 2002, respectively. However, any trade secret or otherwise confidential commercial information in the notifications will not be disclosed to the public.

Prior to October 16, 2002, you may wish to identify in writing specifically what information you believe is proprietary in the notification for Neptune Krill Euphausia™ for FDA's consideration. Nevertheless, our Center's Freedom of Information Officer has the authority to make the final decision about what information in the notifications should be redacted before it is posted at Dockets.

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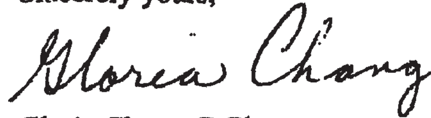
For your information, the following FDA Internet sites and their corresponding links may be useful:
http://www.cfsan.fda.gov/~dms/supplmnt.html
http://www.cfsan.fda.gov/~lrd/fr97923e.html (21 CFR 190.6)
http://www.cfsan.fda.gov/~dms/ds-info.html
http://www.cfsan.fda.gov/~dms/ds-ind.html
http://www.cfsan.fda.gov/~dms/ds-labl.html
http://www.cfsan.fda.gov/~lrd/fr97923b.html
http://www.cfsan.fda.gov/~dms/ds-labl.html#structure
http://www.ftc.gov/bcp/conline/pubs/buspubs/dietsupp.htm

Please contact me at (301) 436-2371, if you have any questions concerning this matter.

Sincerely yours,



Gloria Chang, R.Ph
Interdisciplinary Scientist
HFS-821
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety
and Applied Nutrition

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Laval (Québec)
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Ms. Gloria Chang
Acting Team Leader
Dietary Supplement Team
Division of Standards and Labeling
HFS-821,
CFSAN, FDA
200 C Street, SW
Washington, DC 20204
Telephone No. 301-436-1853

April 26, 2002

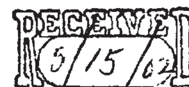
Re: ITPN 79419
Dear Ms. Chang,

Please find enclosed the supplementary information requested.

Please contact me if further information is required at:
tel.: 450-972-6291
cell: 514-865-9917
e-mail: tinas@neptunebiotech.com

Best Regards

Tina Sampalis M.D., Ph.D.
Vice President Research



APPENDIX A : Photostatic copies or reprints of the full-published references,

APPENDIX B : Neptune Krill Oil TM serving and daily dose

APPENDIX C : Neptune Aquateine TM serving and daily dose

APPENDIX D : LyO-Krill daily serving and dose

APPENDIX E : Neptune Krill Enzymes TM serving and daily dose

APPENDIX F : Scientific Report

APPENDIX G : processing techniques used

APPENDIX H : Krill additive by Nippon Suisan

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Neptune Technologies & Bioressources Inc.
Information Package

Table of Contents

1. General information requested
2. Appendix A : Photostatic copies or reprints of the full-published references, citations, and articles in English:
    a. Krill
    b. Cardiovascular disease
    c. Inflammatory disease
    d. Phospholipids
    e. Astaxanthin
3. Appendix B : Neptune Krill Oil™ serving and daily dose
4. Appendix C : Neptune Aquateine™ serving and daily dose
5. Appendix D : LyO-Krill daily serving and dose
6. Appendix E: Neptune Krill Enzymes™ serving and daily dose
7. Appendix F: Scientific Report
8. Appendix G: processing techniques used:
    a. Neptune Krill Oil™ & Neptune Aquateine™
    b. LyO-Krill
9. Appendix H: Krill additive by Nippon Suisan

SUPPLEMENTARY INFORMATION

- The references included abstracts. I informed her that we needed photostatic copies or reprints of the full-published references, citations, and articles in English.
    - Please refer to "Appendix A"

- The specific species of Krill was not identified in the notification. I ask if there was a specific species that was to be marketed. Dr. Sampalis stated that there was, and that she would provide that information to us.
    - The krill species used is: Antarctic Krill *Euphasia superba* fished in the Antarctic ocean near the South Georgia and Sandwich Islands

- The notification was unclear as to what form of the Krill was to be orally ingested, there were 3 forms: the oil extract, the dry fraction, and the freeze-dried form. Dr. Sampalis stated that all three forms were to be marketed for oral ingestion. I asked her to indicate this in her response.
    - All three forms are meant for oral ingestion:
        - Neptune Krill Oil™ : gel caps (1 gram of oil per gel cap)
        - Aquateine™ (dry fraction): powder
        - LyO-Krill (freeze dried): capsules (300mg per capsule)

- **The notification was unclear as to the specific levels or concentration of Krill per serving dose for each form (i.e., the oil extract, the dry fraction, and the freeze-dried Krill) and the total daily serving intake and I asked Dr. Sampalis to provide information for all three forms. I also requested if there was any limitation or duration of use and frequency of serving doses (e.g., how many times a day it is taken), if so, to please indicate, if not to also state this.**
    - Neptune Krill Oil™ : gel caps
        - 1 gram of oil per gel cap
        - recommended dose = 1 – 3 gel caps per day
        - Daily dose: Appendix B

    - Aquateine™ (dry fraction): powder
        - 300mg per sac
        - recommended dose = 1 – 3 sacs per day
        - Daily dose: Appendix C

    - LyO-Krill (freeze dried): capsules
        - 300mg per capsule
        - recommended dose = 1 – 3 capsules per day
        - Daily dose: Appendix D

    - Limitations:
        - No limitation in the duration of use

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- **Because the target population was not clear or missing, to provide the target population that the NDI would be used. If there is no target population, to please indicate that. To also provide information if there is any excluded populations such as pregnant/lactating women, the elderly, children or infants (state age range if any) or other populations with specific disease or medical conditions. If so, to specify, if not also state this.**
  - There is no target population
  - Excluded populations:
    - Seafood allergy
    - Anticoagulant use

- **I also asked Dr. Sampalis to submit the requested complete information in triplicate.**
  - Three copies enclosed

- **Also please make sure that the complete processing information for all three forms, if not in original submission notification, are included in the submission, (i.e., the processing technique used, the chemicals, compounds, or other ingredients used in processing if any, the drying and freezing techniques, etc.)**
  - Please refer to "Appendix E"

- **If there are any other ingredients in the various serving (dosage) forms such as excipients, flavors, coloring, preservatives, stabilizers, etc, also please identify and indicate levels.**
  - The company that fishes the raw material (krill) in the Antarctic add "sodium hydrogen sulfite ($NaHSO_3$)" as an additive upon freezing. The amount of $NaHSO_3$ in krill is approximately 100 ppm.
  - There are no other ingredients included in any of the serving forms

APPENDIX A

MATERIAL SAFETY DATA SHEET

SECTION I - PRODUCT IDENTIFICATION

Product Name: Marine Oil

Product Use: Dietary supplement

WHMIS Class: Not Controlled

TDG Classification: Not Regulated

Manufacturer/Supplier:	**Neptune Technologies & Bioresources**
Address:	500 boul Saint-Martin Ouest Bureau 550
Telephone:	**450 – 972-6291519**

SECTION II – HAZARDOUS INGREDIENTS

Ingredients	CAS#	Wt%	OSHA-PEL	ACGIH-TLV	LC_{50}	LD_{50}

None by WHMIS/OSHA criteria.

SECTION III - PHYSICAL DATA

Boiling Point (°C): Not available
Vapour Pressure (mm Hg): Not available
Vapour Density (Air = 1): Not available
Solubility in Water: Insoluble
Physical State: Liquid
Appearance: Brown

Specific Gravity (H_2O = 1): **Not available**
% Volatile (Wt %): Not available
Evaporation Rate (Ether = 1): Not availab
pH (100%): Not available
Viscosity: Viscous
Odour Threshold (ppm): Not available

SECTION IV - FIRE AND EXPLOSION DATA

Flammability: Not flammable by WHMIS/OSHA criteria.
Flash Point (°C, TCC): None **LEL:** Not applicable **UEL:** Not applicable
Hazardous Combustion Products: May include and are not limited to oxides of carbo
Autoignition Temperature (°C): Not applicable
Means of Extinction: Treat for surrounding material.
Special Fire Hazards: Firefighters should wear self-contained breathing apparatus.

SECTION V - REACTIVITY DATA

Conditions for Chemical Instability: Stable.
Incompatible Materials: None known.
Reactivity, and Under What Conditions: Not available.
Hazardous Decomposition Products: May include and are not limited to oxides of carbon when heated to
Decomposition

SECTION VI - TOXICOLOGICAL PROPERTIES

Route of Entry: Eye, Skin contact, Inhalation, Ingestion.

EFFECTS OF ACUTE EXPOSURE:
Eye: May cause irritation upon direct contact.
Skin: May cause irritation upon direct contact.
Inhalation: May cause respiratory tract irritation.
Ingestion: May cause stomach distress, nausea or vomiting if ingested in large quantities.

EFFECTS OF CHRONIC EXPOSURE:
Skin: Prolonged or repeated exposure can cause drying, defatting and dermatitis.
Irritancy: Non-hazardous by WHMIS/OSHA criteria.
Respiratory Tract Sensitization: No data available.
Carcinogenicity: Non-hazardous by WHMIS/OSHA criteria.
Teratogenicity, Mutagenicity, Reproductive Effects: No data available.
Synergistic Materials: Not available.

SECTION VII- PREVENTATIVE MEASURES

Gloves: No requirements beyond standard industrial hygiene practices.
Eye Protection: No requirements beyond standard industrial hygiene practices.
Respiratory Protection: Not normally required if good ventilation is maintained.
Other Protective Equipment: As required by employer code.
Engineering Controls: General ventilation normally adequate.
Leak and Spill Procedure: Before attempting clean up, refer to hazard data given above. Small spills may be absorbed
with non-reactive absorbent and placed in suitable, covered, labelled containers. Prevent large spills from entering sewers or
waterways. Contact emergency services and supplier for advice.
Waste Disposal: Review federal, state/provincial, and local government requirements prior to disposal.
Storage and Handling Requirements: Keep out of reach of children. Store in a closed container away from incompatible
materials.

SECTION VIII - FIRST AID

Eye: Flush with cool water. Remove contact lenses, if applicable, and continue flushing. Obtain medical attention if irritation
persists.
Skin: Flush with cool water. Wash with soap and water. Obtain medical attention if irritation persists.
Inhalation: If symptoms develop move victim to fresh air. If symptoms persist, obtain medical attention.
Ingestion: Do not induce vomiting. Rinse mouth with water then drink one or two glasses of water. Obtain medical attention.
Never give anything by mouth if victim is unconscious, or is convulsing.

SECTION IX - PREPARATION INFORMATION

Date: 2002/01/24 MSDS Prepared by: KGK SYNERGIZE INC.
Telephone: 1 – 519- 438-9374

Disclaimer
Information for this material safety data sheet was obtained from sources considered technically accurate and reliable. While every effort has been made to ensure full disclosure of product hazards, in some cases data is not available and is so stated. Since conditions of actual product use are beyond control of the supplier, it is assumed that users of this material have been fully trained according to the mandatory requirements of WHMIS. No warranty, expressed or implied, is made and supplier will not be liable for any losses, injuries or consequential damages which may result from the use of or reliance on any information contained in this form. If user requires independent information on ingredients in this or any other material, we recommend contact with the Canadian Centre for Occupational Health and Safety (CCOHS) in Hamilton, Ontario (1-905-572-4400) or CSST in Montreal, Quebec (514-873-3990).

MATERIAL SAFETY DATA SHEET

SECTION I - PRODUCT IDENTIFICATION

Product Name: Krill Protein

Product Use: Dietary supplement

WHMIS Class: Not Controlled

TDG Classification: Not Regulated

Manufacturer/Supplier: Neptune Technologies & Bioresources
Address: 500 boul Saint-Martin Ouest
Bureau 550
Laval, Quebec, H7M 3Y2
Telephone: 450 – 972-6291

SECTION II – HAZARDOUS INGREDIENTS

Ingredients	CAS#	Wt%	OSHA-PEL	ACGIH-TLV	LC 50	LD 50

None by WHMIS/OSHA criteria.

SECTION III - PHYSICAL DATA

Boiling Point (°C): Not applicable
Vapour Pressure (mm Hg): Not applicable
Vapour Density (Air = 1): Not applicable
Solubility in Water: Not available
Physical State: Solid
Appearance: Off-white powder

Specific Gravity (H_2O = 1): Not available
% Volatile (Wt %): Not available
Evaporation Rate (Ether = 1): Not applicable
pH (100%): Not available
Viscosity: Not applicable
Odour Threshold (ppm): Not available

SECTION IV - FIRE AND EXPLOSION DATA

Flammability: Not flammable by WHMIS/OSHA criteria.
Flash Point (°C, TCC): None **LEL:** Not applicable **UEL:** Not applicable
Hazardous Combustion Products: May include and are not limited to oxides of carbon.
Autoignition Temperature (°C): Not applicable
Means of Extinction: Treat for surrounding material.
Special Fire Hazards: Firefighters should wear self-contained breathing apparatus.

SECTION V - REACTIVITY DATA

Conditions for Chemical Instability: Stable.
Incompatible Materials: None known.
Reactivity, and Under What Conditions: Not available.
Hazardous Decomposition Products: May include and are not limited to oxides of carbon when heated to decomposition.

SECTION VI - TOXICOLOGICAL PROPERTIES

Route of Entry: Eye, Skin contact, Inhalation, Ingestion.

EFFECTS OF ACUTE EXPOSURE:
Eye: May cause irritation upon direct contact.
Inhalation: May cause respiratory tract irritation.
Ingestion: May cause stomach distress, nausea or vomiting if ingested in large quantities.

EFFECTS OF CHRONIC EXPOSURE:
Skin: Prolonged or repeated exposure can cause drying, defatting and dermatitis.
Irritancy: Non-hazardous by WHMIS/OSHA criteria.
Respiratory Tract Sensitization: No data available.
Carcinogenicity: Non-hazardous by WHMIS/OSHA criteria.
Teratogenicity, Mutagenicity, Reproductive Effects: No data available.
Synergistic Materials: Not available.

SECTION VII- PREVENTATIVE MEASURES

Gloves: No requirements beyond standard industrial hygiene practices.
Eye Protection: No requirements beyond standard industrial hygiene practices.
Respiratory Protection: Not normally required if good ventilation is maintained.
Other Protective Equipment: As required by employer code.
Engineering Controls: General ventilation normally adequate.
Leak and Spill Procedure: Before attempting clean up, refer to hazard data given above. Use broom or dry vacuum to
collect material for proper disposal without raising dust. Rinse area with water. Prevent large spills from entering sewers or
waterways. Contact emergency services and supplier for advice.
Waste Disposal: Review federal, state/provincial, and local government requirements prior to disposal.
Storage and Handling Requirements: Keep out of reach of children. Store in a closed container away from incompatible
materials.

SECTION VIII - FIRST AID

Eye: Flush with cool water. Remove contact lenses, if applicable, and continue flushing. Obtain medical attention if irritation
persists.
Skin: Brush away excess of dry material. Flush with water. Obtain medical attention if irritation persists.
Inhalation: If symptoms develop move victim to fresh air. If symptoms persist, obtain medical attention.
Ingestion: Do not induce vomiting. Rinse mouth with water then drink one or two glasses of water. Obtain medical attention.
Never give anything by mouth if victim is unconscious, or is convulsing.

SECTION IX - PREPARATION INFORMATION

Date: 2002/01/24 **MSDS Prepared by: KGK SYNERGIZE INC.**
Telephone: 1 – 519 –438-9374

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MATERIAL SAFETY DATA SHEET

SECTION I - PRODUCT IDENTIFICATION

Product Name: Freeze Dried Krill

Product Use: Dietary supplement

WHMIS Class: Not Controlled

TDG Classification: Not Regulated

Manufacturer/Supplier: Neptune Technologies & Bioresources
Address: 500 boul Saint-Martin Ouest
Bureau 550
Laval, Quebec, H7M 3Y2
Telephone: 450 – 972-6291

SECTION II – HAZARDOUS INGREDIENTS

Ingredients	CAS#	Wt%	OSHA-PEL	ACGIH-TLV	LC 50	LD 50

None by WHMIS/OSHA criteria.

SECTION III - PHYSICAL DATA

Boiling Point (°C): Not applicable
Vapour Pressure (mm Hg): Not applicable
Vapour Density (Air = 1): Not applicable
Solubility in Water: Not available
Physical State: Solid
Appearance: Off-white powder

Specific Gravity (H2O = 1): Not available
% Volatile (Wt %): Not available
Evaporation Rate (Ether = 1): Not applicable
pH (100%): Not available
Viscosity: Not applicable
Odour Threshold (ppm): Not available

SECTION IV - FIRE AND EXPLOSION DATA

Flammability: Not flammable by WHMIS/OSHA criteria.
Flash Point (°C, TCC): None **LEL:** Not applicable **UEL:** Not applicable
Hazardous Combustion Products: May include and are not limited to oxides of carbon.
Autoignition Temperature (°C): Not applicable
Means of Extinction: Treat for surrounding material.
Special Fire Hazards: Firefighters should wear self-contained breathing apparatus.

SECTION V - REACTIVITY DATA

Conditions for Chemical Instability: Stable.
Incompatible Materials: None known.
Reactivity, and Under What Conditions: Not available.
Hazardous Decomposition Products: May include and are not limited to oxides of carbon when heated to decomposition.

SECTION VI - TOXICOLOGICAL PROPERTIES

Route of Entry: Eye, Skin contact, Inhalation, Ingestion.

EFFECTS OF ACUTE EXPOSURE:
Eye: May cause irritation upon direct contact.
Inhalation: May cause respiratory tract irritation.
Ingestion: May cause stomach distress, nausea or vomiting if ingested in large quantities.

EFFECTS OF CHRONIC EXPOSURE:
Skin: Prolonged or repeated exposure can cause drying, defatting and dermatitis.
Irritancy: Non-hazardous by WHMIS/OSHA criteria.
Respiratory Tract Sensitization: No data available.
Carcinogenicity: Non-hazardous by WHMIS/OSHA criteria.
Teratogenicity, Mutagenicity, Reproductive Effects: No data available.
Synergistic Materials: Not available.

SECTION VII- PREVENTATIVE MEASURES

Gloves: No requirements beyond standard industrial hygiene practices.
Eye Protection: No requirements beyond standard industrial hygiene practices.
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Other Protective Equipment: As required by employer code.
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waterways. Contact emergency services and supplier for advice.
Waste Disposal: Review federal, state/provincial, and local government requirements prior to disposal.
Storage and Handling Requirements: Keep out of reach of children. Store in a closed container away from incompatible
materials.

SECTION VIII - FIRST AID

Eye: Flush with cool water. Remove contact lenses, if applicable, and continue flushing. Obtain medical attention if irritation
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Inhalation: If symptoms develop move victim to fresh air. If symptoms persist, obtain medical attention.
Ingestion: Do not induce vomiting. Rinse mouth with water then drink one or two glasses of water. Obtain medical attention.
Never give anything by mouth if victim is unconscious, or is convulsing.

SECTION IX - PREPARATION INFORMATION

Date: 2002/01/24 **MSDS Prepared by: KGK SYNERGIZE INC.**
Telephone: 1 – 519 –438-9374

Disclaimer
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[Code of Federal Regulations]
[Title 21, Volume 1, Parts 1 to 99]
[Revised as of April 1, 2000]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR73.75]

[Page 337-338]

TITLE 21--FOOD AND DRUGS

PART 73--LISTING OF COLOR ADDITIVES EXEMPT FROM CERTIFICATION--Table of Contents

Subpart A--Foods

Sec. 73.75 Canthaxanthin.

(a) Identity. (1) The color additive canthaxanthin is <greek-b>-carotene-4,4'-dione.

(2) Color additive mixtures for food use made with canthaxanthin may contain only those diluents that are suitable and that are listed in this subpart as safe for use in color additive mixtures for coloring foods.

(b) Specifications. Canthaxanthin shall conform to the following specifications and shall be free from impurities other than those named to the extent that such other impurities may be avoided by good manufacturing practice:

Physical state, solid.
1 percent solution in chloroform, complete and clear.
Melting range (decomposition), 207 deg.C. to 212 deg.C. (corrected).
Loss on drying, not more than 0.2 percent.
Residue on ignition, not more than 0.2 percent.
Total carotenoids other than trans-canthaxanthin, not more than 5 percent.
Lead, not more than 10 parts per million.
Arsenic, not more than 3 parts per million.
Mercury, not more than 1 part per million.
Assay, 96 to 101 percent.

(c) Use and restrictions. (1) The color additive canthaxanthin may be safely

[[Page 338]]

used for coloring foods generally subject to the following restrictions:

(i) The quantity of canthaxanthin does not exceed 30 milligrams per pound of solid or semisolid food or per pint of liquid food; and

(ii) It may not be used to color foods for which standards of identity have been promulgated under section 401 of the act unless added color is authorized by such standards.

(2) Canthaxanthin may be safely used in broiler chicken feed to enhance the yellow color of broiler chicken skin in accordance with the

following conditions: The quantity of canthaxanthin incorporated in the feed shall not exceed 4.41 milligrams per kilogam (4 grams per ton) of complete feed to supplement other known sources of xanthophyll and associated carotenoids to accomplish the intended effect.

(3) Canthaxanthin may be safely used in the feed of salmonid fish in accordance with the following prescribed conditions:

(i) Canthaxanthin may be added to the fish feed only in the form of a stabilized color additive mixture;

(ii) The color additive is used to enhance the pink to orange-red color of the flesh of salmonid fish; and

(iii) The quantity of color additive in feed shall not exceed 80 milligrams per kilogram (72 grams per ton) of finished feed.

(d) Labeling requirements. (1) The labeling of the color additive and any mixture prepared therefrom intended solely or in part for coloring purposes shall conform to the requirements of Sec. 70.25 of this chapter.

(2) For purposes of coloring fish, the labeling of the color additive and any premixes prepared therefrom shall bear expiration dates (established through generally accepted stability testing methods) for the sealed and open container, other information required by Sec. 70.25 of this chapter, and adequate directions to prepare a final product complying with the limitations prescribed in paragraph (c)(3) of this section.

(3) The presence of the color additive in finished fish feed prepared according to paragraph (c)(3) of this section shall be declared in accordance with Sec. 501.4 of this chapter.

(4) The presence of the color additive in salmonid fish that have been fed feeds containing canthaxanthin shall be declared in accordance with Secs. 101.22(b), (c), and (k)(2), and 101.100(a)(2) of this chapter.

(e) Exemption from certification. Certification of this color additive is not necessary for the protection of the public health, and therefore batches thereof are exempt from the certification requirements of section 721(c) of the act.

[42 FR 15643, Mar. 22, 1977, as amended at 50 FR 47534, Nov. 19, 1985; 63 FR 14817, Mar. 27, 1998]

Color Additive Regulations

Foods Home | FDA Home | HHS Home | Search/Subject Index | Disclaimers & Privacy Policy

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[Code of Federal Regulations]
[Title 21, Volume 1, Parts 1 to 99]
[Revised as of April 1, 2000]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR73.1075]

[Page 352]

TITLE 21--FOOD AND DRUGS

PART 73--LISTING OF COLOR ADDITIVES EXEMPT FROM CERTIFICATION--Table of Contents

Subpart B--Drugs

Sec. 73.1075 Canthaxanthin.

(a) Identity and specifications. (1) The color additive canthaxanthin shall conform in identity and specifications to the requirements of Sec. 73.75(a)(1) and (b).

(2) Color additive mixtures for ingested drug use made with canthaxanthin may contain only those diluents that are suitable and that are listed in this subpart as safe in color additive mixtures for coloring ingested drugs.

(b) Uses and restrictions. Canthaxanthin may be safely used for coloring ingested drugs generally in amounts consistent with good manufacturing practice.

(c) Labeling requirements. The label of the color additive and of any mixtures prepared therefrom intended solely or in part for coloring purposes shall conform to the requirements of Sec. 70.25 of this chapter.

(d) Exemption from certification. Certification of this color additive is not necessary for the protection of the public health, and therefore batches thereof are exempt from the certification requirements of section 721(c) of the act.

This electronic document was downloaded from the GPO web site, May 2001, and is provided for information purposes only. The Code of Federal Regulations, Title 21, is updated each year in early summer. The most current version of the regulations may be found at the GPO web site or from the current printed version.

[Code of Federal Regulations]
[Title 21, Volume 1, Parts 1 to 99]
[Revised as of April 1, 2000]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR73.35]

[Page 336]

TITLE 21--FOOD AND DRUGS

PART 73--LISTING OF COLOR ADDITIVES EXEMPT FROM CERTIFICATION--Table of Contents

Subpart A--Foods

Sec. 73.35 Astaxanthin.

(a) Identity. (1) The color additive astaxanthin is 3, 3'-dihydroxy-<greek-b>, <greek-b>-carotene-4, 4'-dione.

(2) Astaxanthin may be added to the fish feed only as a component of a stabilized color additive mixture. Color additive mixtures for fish feed use made with astaxanthin may contain only those diluents that are suitable and are listed in this subpart as safe for use in color additive mixtures for coloring foods.

(b) Specifications. Astaxanthin shall conform to the following specifications and shall be free from impurities other than those named to the extent that such impurities may be avoided by good manufacturing practice:

Physical state, solid.
0.05 percent solution in chloroform, complete and clear.
Absorption maximum wavelength 484-493 nanometers (in chloroform).
Residue on ignition, not more than 0.1 percent.
Total carotenoids other than astaxanthin, not more than 4 percent.
Lead, not more than 5 parts per million.
Arsenic, not more than 2 parts per million.
Mercury, not more than 1 part per million.
Heavy metals, not more than 10 parts per million.
Assay, minimum 96 percent.

(c) Uses and restrictions. Astaxanthin may be safely used in the feed of salmonid fish in accordance with the following prescribed conditions:

(1) The color additive is used to enhance the pink to orange-red color of the flesh of salmonid fish.

(2) The quantity of color additive in feed is such that the color additive shall not exceed 80 milligrams per kilogram (72 grams per ton) of finished feed.

(d) Labeling requirements. (1) The labeling of the color additive and any premixes prepared therefrom shall bear expiration dates for the sealed and open container (established through generally accepted stability testing methods), other information required by Sec. 70.25 of this chapter, and adequate directions to prepare a final product

complying with the limitations prescribed in paragraph (c) of this section.

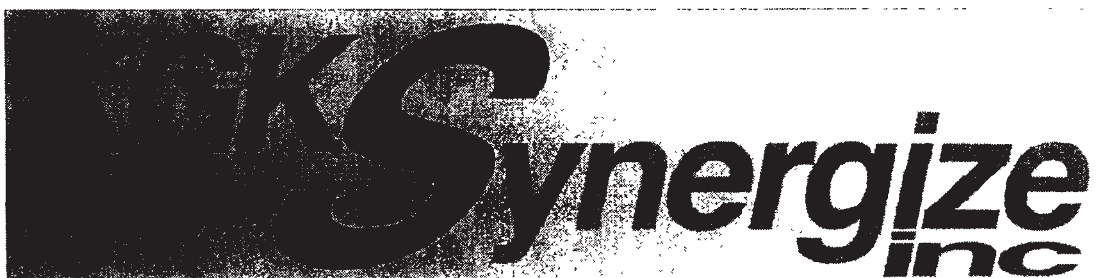
(2) The presence of the color additive in finished fish feed prepared according to paragraph (c) of this section shall be declared in accordance with Sec. 501.4 of this chapter.

(3) The presence of the color additive in salmonid fish that have been fed feeds containing astaxanthin shall be declared in accordance with Secs. 101.22(k)(2) and 101.100(a)(2) of this chapter.

(e) Exemption from certification. Certification of this color additive is not necessary for the protection of the public health, and therefore batches thereof are exempt from the certification requirements of section 721(c) of the act.

[60 FR 18738, Apr. 13, 1995]

[[Page 337]]



ANALYTICAL REPORT

Client	Louis Lappointte
Company	Neptune Technologies & Bioressources Inc.
Date Received	November 14, 2001.
Date Reported	November 22, 2001.

Analysis of astaxanthin and canthaxanthin was performed on the following samples submitted with your order

Sample name	**Type of sample**
800	Marine oil
801	Marine oil

The following reference method was used

Enzymatic hydrolysis using Lipase from Candida rugosa followed by extraction and high performance liquid chromatography.

References
Xinia el al. J, Food Comp. Anal. 13 (2000), 179-187.

	800	801
Canthaxanthin µg/g	389.3	454.2
Astaxanthin µg/g	168.7	122.3

This report has been approved by:

Elzbieta M. Kurowska, Ph.D.
Vice President, Research & Development

Suite 1030, One London Place, 255 Queens Avenue, London, ON N6A 5R8 Canada Tel: (519) 438-9374 or (519) 438-8916 Fax: (519) 438-8314 E-mail: kurowska@kgksynergize.com www.kgksynergize.com

AKER877ITC00057934

RX-0135.0029

AKER877ITC00057935

RX-0135.0030

ANALYTICAL REPORT

Client	Louis Lappointte
Company	Neptune Technologies & Bioressources Inc.
Date Received	November 14, 2001.
Date Reported	November 23, 2001.

Trace metal analysis was performed on the following samples submitted with your order

Sample name	**Type of sample**
800	Marine oil
801	Marine oil

The following reference method has been used

Digestion by nitric acid and hydrogen peroxide followed by ICP-MS (Inductively Coupled Plasma Mass Spectrometry)

This report has been approved by:

Elzbieta M. Kurowska, Ph.D.
Vice President, Research & Development

Suite 1030, One London Place, 255 Queens Avenue, London, ON N6A 5R8 Canada Tel: (519) 438-9374 or (519) 438-8916 Fax: (519) 438-8314 E-mail: kurowska@kgksynergize.com www.kgksynergize.com

	800	801
Lead μg/g	0.005	0.002
Sodium mg/g	0.0023	0.019
Magnesium μg/g	0.06	0.03
Iron μg/g	0.87	0.41
Copper μg/g	0.05	0.09
Zinc μg/g	0.06	0.04
Selenium μg/g	0.05	0.02
Potassium mg/g	0.015	0.024

AKER877ITC00057937

RX-0135.0032

AKER877ITC00057938

RX-0135.0033

BIOPHARM inc.

3885, boul. Industriel,Laval Québec
Canada H7L 4S3
Tél. (450) 663-6724
Téléc. (450) 975-8111

Certificate of analysis

NCA : N18-020408-37
Req : -
Code Client : N18
Version : 1

Our quality system is certified according to ISO-9002 standard

Printed : 2002/04/29
Received : 2002/04/08
Date : 2002/04/29
(yyyy/mm/dd)

Neptune Technologies/Tina Sampalis
500, boul. St-Martin Ouest
Laval, Québec H7M 3Y2

RESULT

Sample : MARINE OIL

Section : 1
Lot Not : WA1303
Gabarit : Raw material
Description : Reddish opaque oily liquid having a fishy odor — Complies

TEST	METHOD	SPECIFICATION	RESULT
Cholesterol	JAOAC Vol. 76, No. 4 (GLC)	Report (g/100 g)	1.3
Vitamin A	HPLC	Report (UI/g)	809.2
Vitamin E	HPLC	Report (UI/g)	1 01
Free fatty acid profile	AOCS Ce 1b-89 (GLC)	Report (%)	(2)
A[illegible]e	GLC	Report (ppm)	< 100
Free fatty acids	USP <401> (1)	Report (mg KOH/g)	29.7
Phosphatidyl inositol	(TLC)	Report (g/100 g)	< 2 5
Phosphatidyl choline	(TLC)	Report (g/100 g)	5 0
Phosphatidyl ethanolamine	(TLC)	Report (g/100 g)	48 5
Phosphatidyl serine	(TLC)	Report (g/100 g)	< 2 5
Sphingomyelin	(TLC)	Report (g/100 g)	< 2 4
Total phospholipids	Colorimetry	Report (g/100 g)	53.5
Iodine value	A.O.C.S. Cd. 1-25	Report (mg I/100g)	3 56
Oil stability index	A.O.C.S. Cd. 12b. 92	Induction time converted to the reference temperature of 97.8°C (hrs)	> 50
p-Anidisine value	A O.C S. Cd. 18 90	Report	1 98
Peroxide value	A.O C.S. Cd. 8b. 90	Report (meq peroxide/kg)	< 0 1
Saponification index	A.O C S. Cd 3.25	Report (mg KOH/g)	172 1
Moisture and volatile matter	Dried 16 h at 60°C under vacuum	Report (%)	0 61

(1) Under Acid Value, (2) (100 - % ash), USP Current Edition

Verified by : **Sylvain Mandeville, Ph.D., Scientific Director**

Approved by : **Lucie Blanchard, Chemist**

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BIOPHARM inc.

3885, boul. Industriel,Laval Québec
Canada H7L 4S3
Tél. (450) 663-6724
Téléc. (450) 975-8111

Certificate of analysis

NCA : N18-020408-37
Req : -
Code Client : N18
Version : 1

Our quality system is certified according to ISO-9002 standard

Printed : 2002/04/29
Received : 2002/04/08
Date : 2002/04/29
(yyyy/mm/dd)

Neptune Technologies/Tina Sampalis
500, boul. St-Martin Ouest
Laval, Québec H7M 3Y2

Sample : MARINE OIL

			RESULT
Viscosity	FP-78	Report (cst)	927.2
Ash (total inorganic substances)	KGK (2h at 600°C)	Report (%)	4.09
Total protein	CA-126-050	Report (%)	6.08
Total lipids	CG-118-039	Report (%)	79.3
Assay of Sodium (AAS)	CM-109-125	Report (mg/100 g)	386
Assay of Zinc (AAS)	CM-109-125	Report (mg/100 g)	0.66
Assay of Potassium (AAS)	CM-109-125	Report (mg/100 g)	207
Assay of Calcium (AAS)	CM-109-125	Report (mg/100 g)	0.60
Assay of Selenium (AAS)	CM-109-215	Report (mg/100 g)	< 3 1
/ of Aluminium (AA)	CM-109-125	Report (mg/100 g)	7.45
As.ay of Copper (AA)	CM-109-125	Report (mg/100 g)	0.17
Assay of Manganese (AAS)	CM-109-125	Report (mg/100 g)	< 0.31

(1) Under Acid Value, (2) (100 - % ash), USP Current Edition

Verified by : **Sylvain Mandeville, Ph.D., Scientific Director**

Approved by : **Lucie Blanchard, Chemist**

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CERTIFICATE OF ANALYSIS

NCA: N18-020408-37
Req: -
Code client: N18
Version: 1

Neptune Technologies
500, boul. St-Martin Ouest
Laval, Québec H7M 3Y2

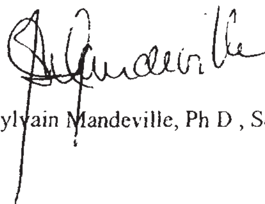
Reçu: 2002/04/08
Date: 2002/04/29
Section: 2

Échantillon: Marine Oil, lot WA1303

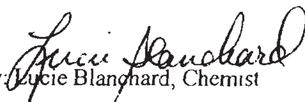
FATTY ACIDS	AREA %	g/100 g of sample
C 14 :0	6.10	4.84
C 14 :1	0.15	0.12
C 15 :0	0.34	0.27
C 16 :0	19.55	15.50
C 16 :1	5.89	4.67
C 18 :0	0.82	0.65
C 18 :1	12.77	10.13
C 18 :2n-6	1.47	1.17
C 18 :4n-6	0.05	0.04
C 18 :3n-3	0.72	0.57
C 18 :4n-3	1.11	0.88
C 20 :0	0.08	0.06
C 20 :1	0.38	0.30
C 20 :2n-6	0.13	0.10
C 20 :4n-6	0.65	0.52
C 20 :4n-3	0.47	0.37

3885, boul. Industriel, Laval (Quebec), Canada H7L 4S3
Tél : (450) 663-6724 Fax. (450) 669-2784 www.warnex.ca

C 20 :5n-3 (EPA)	23.06	18.30
C 22 :0	0.25	0.20
C 21 :5n-3	7.12	5.64
C 23 :0	1.21	0.96
C 22 :5n-3	0.12	0.09
C 24 :0	1.09	0.87
C 22 :6n-3	12.09	9.58
C 24 :1	0.32	0.25



Verified by: Sylvain Mandeville, Ph D , Scientific Director



Approved by: Lucie Blanchard, Chemist

SGS Laboratoires d'Analyses Agro-Alimentaires
Une Division de SGS Canada Inc.

185 boul Brunswick
Pointe-Claire, Québec
Canada H9R 4Z1
Téléphone (514) 630-6098
Télécopieur (514) 630-6095

TEST REPORT

NEPTUNE TECHNOLOGIES & BIORESSOURCES INC.
500, St-Martin ouest, bureau 550
Laval, Québec
H7M 3Y2

ATTENTION OF M. LUC RAINVILLE

RECEPTION DATE : 01 11 16
LABORATORY NUMBER : 160080544R
NUMBER OF SAMPLES : 5
REPORT DATE : 01 12 05
PAGE : 1 DE 1
ANALYSIS REPORT : 01 11 30

SAMPLES		1 KRILL 15-11-01	2 KRILL 15-11-01	3 KRILL 15-11-01	4 KRILL 15-11-01	5 KRILL 15-11-01	
ANALYSIS							
ACIDITY (CITRIC ACID)	G/100G	0.02	0.03	0.03	0.03	0.03	0,028
INTERSPACE WATER	G/100G	9.48	9.43	9.61	16.96	11.48	
SULFITES	PPM	< 2	< 2	< 2	< 2	< 2	<2
MOISTURE	G/100G	81.9	81.2	80.8	80.9	80.9	81,14
ASH	G/100G	3.0	2.9	3.0	3.0	3.0	2,98
PROTEINS	G/100G	13.2	13.6	13.6	14.2	14.1	13.74
FAT	G/100G	2.0	2.3	2.2	2.3	2.5	2.26
CARBOHYDRATES	G/100G	0.0	0.0	0.3	0.0	0.0	0,06
ENERGY PER 100 G	CALORIES	71	75	75	78	78	75.4

J. Beaupré

JACQUELINE BEAUPRÉ, SUPERVISEURE - CHIMIE   01 12 05   RÉVISION 1
neptuneCHEMmas
FAX : 450-972-6351
FAX: 450-979-0660

This report is issued by the Company under its General Conditions for Inspection and Testing Services (copy available upon request). The issuance of this report does not exonerate buyers or sellers from exercising all their rights and discharging all their liabilities under the Contract of Sale. Stipulations to the contrary are not binding on the Company. The Company's responsibility under this report is limited to proven negligence and will in no case be more than ten times the amount of the fees or commission. Except by special arrangement, samples, if drawn, will not be retained by the Company for more than one month.

LYO-SAN Inc.
500, boul. de l'Aéroparc, C.P. 598
Lachute (Québec) Canada. J8H 4G4

lyosan

Tél.: (514) 562-8525
Fax: (514) 562-1433

RAPPORT D'ANALYSE

Date 25 février 2002

No de lot 12-2-02

Nom du produit : Krill lyophilisé en poudre

Description : Poudre couleur rosée, forte odeur de poisson.

Conditions d'entreposage :

TESTS	METHODES	SPECIFICATIONS	RESULTATS
Humidité	O'Haus	< 5%	1.1%
Microbiologie			
Compte Total	U.S.P.		500/g
Levures Moisissures	U.S.P.		< 100/g
Coliformes Totaux	A.P.H.A.		< 10/g
S. Aureus	A.P.H.A.		< 100/g
E. Coli 0157	U.S.P.		absence
Salmonella sp.	U.S.P.		absence

Analyste : [signature]

MANUFACTURIER DE PRODUITS LYOPHILISÉS (SECHÉS À FROID) & DE LYOPHILISATEURS.
FREEZE DRIED FOODS & FREEZE DRYERS MANUFACTURER.

AKER877ITC00057945

RX-0135.0040

SGS Laboratoires d'Analyses Agro-Alimentaires
Une Division de SGS Canada Inc.

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Pointe-Claire, Québec
Canada H9R 4Z1
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Télécopieur (514) 630-6095

NEPTUNE TECHNOLOGIES & BIORESSOURCES INC.
500, St-Martin ouest, bureau 550
Laval, Québec
H7M 3Y2

À L'ATTENTION DE MME. TINA SAMPALIS

DATE DE RÉCEPTION: 02-03-06
NUMERO DE LABORATOIRE: 160083519
NOMBRE D'ÉCHANTILLONS: 2
DATE DE RAPPORT: 02 03 26

DATE D'ANALYSE: 02-03-06

	ÉCHANTILLONS:	1 A-SGS-1 EXTRACTION #114, 44IÈME SÉCHAGE DU 14-02-2002, EMBALÉ LE 15-02-2002	2 LK-SGS-1 KRILL LYO EN POUDRE, LOT: 180202 POIDS NET 250G
	MÉTHODE:		
BACTÉRIES TOTALES /G	MFHPB-33	<100	1,400
COLIFORMES TOTAUX /G	MFHPB-34	<10	<10
E.COLI /G	MFHPB-34	<10	<10
STAPH. AUREUS /G	MFHPB-21	<10	<10
LEVURES /G	MFHPB-22	<10	<10
MOISSISURES /G	MFHPB-22	<10	<10
SALMONELLES /G	MFHPB-20	NON DÉTECTÉE	NON DÉTECTÉE
PSEUDOMONAS SPP.		<50	50

D. Beaupré pour.

DANIÈLE LESSARD
SUPERVISEURE DES SERVICES TECHNIQUES ET DU CQ 02 03 26
neptuneMICmas
FAX: 450-972-6351

Le présent rapport a été émis par la Société conformément à ses Conditions Générales pour les prestations de services de contrôle et d'analyse (copie disponible sur demande) L'émission du présent rapport ne dispense pas les acheteurs ou les vendeurs d'exercer tous leurs droits et d'exécuter toutes leurs obligations liés au contrat de vente. Toute stipulations contraires n'engagent pas la Société. La responsabilité de la Société relative au présent rapport est limitée à la négligence prouvée et n'excèdera en aucun cas dix fois le montant des honoraires ou de la commission. Sauf disposition spéciale, les échantillons, s'il en a été prélevés, ne seront pas conservés par la Société au delà d'une période de un mois.

SGS Agri-Food Testing Laboratories
A Division of SGS Canada Inc.

RAPPORT D'ESSAIS

185 Brunswick Blvd
Pointe-Claire, Quebec
Canada H9R 4Z1
Telephone (514) 630-6093
Fax (514) 630-6095

NEPTUNE TECHNOLOGIES & BIORESSOURCES INC.
500, St-Martin ouest, bureau 550
Laval, Québec
H7M 3Y2

À L'ATTENTION DE MME. TINA SAMPALIS

DATE DE RÉCEPTION: 02 03 06
NUMERO DE LABORATOIRE: 160083519
NOMBRE D'ÉCHANTILLONS: 2
DATE DE RAPPORT: 02 03 26
PAGE: 5 DE 7
DATE D'ANALYSE: 02 03 11

PRODUIT: CONCENTRÉ DE PROTÉINES A-SGS-1

	DESCRIPTION	CONTENU uMOL/gr	CONTENU mg/gr	%
1	TAURINE	81	10.1	2.15
2	AC. ASPARTIQUE	312	41.5	8.81
3	HYDROXYPROLINE			
4	THRÉONINE	188	22.4	4.75
5	SÉRINE	120	12.6	2.68
6	ASPARAGINE			
7	AC. GLUTAMIQUE	392	57.7	12.24
8	GLUTAMINE			
9	SARCOSINE			
10	AC. AMINOADIPIQUE			
11	PROLINE	199	22.9	4.86
12	GLYCINE	329	24.7	5.24
13	ALANINE	282	25.1	5.33
14	CITRULLINE	1		
15	AC. AMINO-n-BUTYRIQUE			
16	VALINE	211	24.7	5.24
17	CYSTINE	30	7.2	1.53
18	MÉTHIONINE	80	11.9	2.53
19	HOMOCITRULLINE			
20	CYSTATHIONINE			
21	ISOLEUCINE	161	21.1	4.48
22	LEUCINE	269	35.3	7.49
23	AC.ARGININOSUCCINIQUE			
24	TYROSINE	131	23.7	5.04
25	B-ALANINE			
26	PHÉNYLALANINE	165	27.3	5.78
27	AC. B-AMINO-ISOBUTYRIQUE			
28	HOMOCYSTINE			
29	AC Y-AMINOBUTYRIQUE			
30	ORNITINE	46	6.1	1.29
31	LYSINE	282	41.2	8.75
32	1-MÉTHYLHISTIDINE			
33	HISTIDINE	69	10.7	2.27
34	3-MÉTHYLHISTIDINE			
35	CARNOSINE			
36	ARGININE	218	38.0	8.06
37	TRYPTOPHANE	34	6.9	1.47
	Total	3600	471.3	100 0

J. Beaupré

JACQUELINE BEAUPRÉ, SUPERVISEURE - CHIMIE 02 03 26
neptuneCHEMmas
FAX: 450-972-6351

Le présent rapport a été émis par la Société conformément à ses Conditions Générales pour les prestations de services de contrôle et d'analyse (copie disponible sur demande). L'émission du présent rapport ne dispense pas les acheteurs ou les vendeurs d'exercer tous leurs droits et d'exécuter toutes leurs obligations liés au contrat de vente. Toute stipulations contraires n'engagent pas la Société. La responsabilité de la Société relative au présent rapport est limitée à la négligence prouvée et n'excédera en aucun cas dix fois le montant des honoraires ou de la commission. Sauf disposition spéciale, les échantillons, s'il en a été prélevés, ne seront pas conservés par la Société au delà d'une période de un mois

SGS Laboratoires d'Analyses Agro-Alimentaires
Une Division de SGS Canada Inc.

185 boul. Brunswick
Pointe-Claire, Québec
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Téléphone (514) 630-6093
Télécopieur (514) 630-6095

RAPPORT D'ESSAIS

NEPTUNE TECHNOLOGIES & BIORESSOURCES INC.
500, St-Martin ouest, bureau 550
Laval, Québec
H7M 3Y2

À L'ATTENTION DE MME. TINA SAMPALIS

DATE DE RÉCEPTION: 02 03 06
NUMERO DE LABORATOIRE: 160083519
NOMBRE D'ÉCHANTILLONS: 2
DATE DE RAPPORT: 02 03 26
PAGE: 4 DE 7
DATE D'ANALYSE: 02 03 11

ÉCHANTILLONS		SPECIFICATIONS	1 CONCENTRÉ DE PROTÉINES A-SGS-1	2 CONCENTRÉ DE PROTÉINES LK-SGS-1
ANALYSES				
ALUMINIUM	PPM		46	43
BARIUM	PPM		5.0	4.6
BORON	PPM		21	18
CADMIUM	PPM		0.6	0.4
CALCIUM	PPM		22600	17300
CHROMIUM	PPM		1.1	1.2
COPPER	PPM		114	92.2
FLUORIDE	PPM		376	411
IRON	PPM		54.6	45.2
LEAD	PPM		< 1	< 1
MAGNESIUM	PPM		6940	5010
MANGANESE	PPM		2.8	2.2
MERCURY	PPM		< 0.1	< 0.1
PHOSPHORUS	PPM		15200	13500
POTASSIUM	PPM		8290	14010
SELENIUM	PPM		5.2	4.4
SODIUM	PPM		19600	29300
STRONTIUM	PPM		346	260
SULPHUR	PPM		12600	11500
ZINC	PPM		74	53

J. Beaupré

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RAPPORT D'ESSAIS

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ÉCHANTILLONS		SPECIFICATIONS	1 CONCENTRÉ DE PROTÉINES A-SGS-1	2 CONCENTRÉ DE PROTÉINES LK-SGS-1
ANALYSES				
CHOLESTEROL	MG/100G		26	528
SATURATED FATTY ACIDS	G/100G		0.42	1.93
MONOUNSATURATED	G/100G		0.38	1.68
POLYUNSATURATED	G/100G		0.33	1.95
TRANS	G/100G		< 0.01	< 0.01
EPA	G/100G		0.15	0.99
DHA	G/100G		0.14	0.62
PC	G/100G		0.31	3.32
PI	G/100G		0.04	0.04
PS	G/100G		0.05	0.07
PE	G/100G		0.16	0.58
SM	G/100G		0.05	0.16

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ÉCHANTILLONS		SPECIFICATIONS	1 CONCENTRÉ DE PROTÉINES A-SGS-1	2 CONCENTRÉ DE PROTÉINES LK-SGS-1
ANALYSES				
MOISTURE	G/100G		2.76	2.92
FAT	G/100G		1.13	5.54
PROTEIN	G/100G		81.71	73.39
ASH	G/100G		14.07	15.77
CARBOHYDRATES	G/100G		0.33	2.38
ENERGY	CAL/100G		338	353
ENERGY	KJ/100G		1436	1493
OXYCAROTENOIDS*	MG/100G		0.7	11.0
ACETONE	PPM		300	--

* Astaxanthine & canthaxanthine

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DATE DE RAPPORT: 02 05 08
PAGE: 3 DE 7
DATE D'ANALYSE: 02 03 11

ÉCHANTILLONS		SPECIFICATIONS	1 CONCENTRÉ DE PROTÉINES A-SGS-1	2 CONCENTRÉ DE PROTÉINES LK-SGS-1
ANALYSES				
VITAMIN A (RETINOL)	UI/100G		275	7100
VITAMIN E	UI/100G		16	82
VITAMIN D	UI/100G		<10	<10
RIBOFLAVIN (B2)	MG/100G		6.6	23.6
THIAMINE (B1)	MG/100G		< 1	< 1
VITAMIN H6	MG/100G		< 0.1	< 0.1
CYANOCOBALAMINE (B12)	UG/100G		< 10	< 10
FOLIC ACID	MG/100G		0.2	1.7
PANTHOTENIC ACID	MG/100G		102	676
BIOTIN	PPM		92	<10
NIACIN	MG/100G		67.6	187.3

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Aquasearch, Inc. **Technical Report**

Haematococcus Pluvialis and Astaxanthin Safety For Human Consumption

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Safety for human consumption of *Haematococcus pluvialis* algal meal and astaxanthin has been demonstrated by a number of studies:

- **A recent 28-day rat study with *Haematococcus pluvialis* dry algal meal, produced by Aquasearch's proprietary technology, demonstrated that there was no observed sub-acute toxicity at a daily dose of 50 mg/kg body weight, corresponding to 3,500 mg algal meal per 70-kg body weight of a typical adult man.**
- **No lethality was seen for *Haematococcus pluvialis* algae at doses up to 5000 mg/kg body weight, in an earlier, 13-day, single-dose (acute-toxicity), rat study.**
- **A human safety study demonstrated that daily ingestion of up to 1,140 mg Aquasearch's *Haematococcus pluvialis* algal meal, for 29 days, did not result in any safety concern.**
- **A recent sub-acute rat toxicity with Aquasearch's *Haematococcus pluvialis* algal meal, showed no signs of toxicity, after dosing rats with up to 1.15 mg astaxanthin per kg body weight per day (equivalent to 80.5 mg astaxanthin per 70-kg body weight) for 28 consecutive days.**
- **In a human safety study with Aquasearch's algal astaxanthin, no sign of toxicity or safety concern was observed, when volunteers ingested up to 19.25 mg astaxanthin per day for 29 days, while an earlier human study failed to find any harmful effect from 14.4 mg/day astaxanthin ingestion for two weeks.**
- **Pure astaxanthin (up to 80 mg/kg feed), is Generally Considered As Safe by FDA, for use in salmon diets. This can result in astaxanthin deposition of 10 to 15 mg/kg in salmon fillets. Levels of astaxanthin naturally occurring in wild-caught seafood, and dietary studies on carotenoids, seafood, and salmon,**

also suggest that a daily serving of 5 mg astaxanthin, corresponding to 125 g of wild-caught Sockeye salmon fillet or less than 100 g of krill, is safe.

- **The proprietary technology and quality control developed by Aquasearch to produce *Haematococcus pluvialis* algal meal, ensure that the product meets dietary supplement safety standards.**

Conclusion: A supplement containing 5 mg astaxanthin derived from 250 mg, or less, of Aquasearch's *Haematococcus pluvialis* algal meal is safe for daily human consumption.

Aquasearch's proprietary technology allows the production of a high quality algal meal containing 2% total astaxanthin or more. It is therefore a very good source of natural astaxanthin, a carotenoid pigment and biological antioxidant widely encountered in nature. Safety for human consumption of astaxanthin and ***Haematococcus pluvialis*** algae has been demonstrated by a number of studies.

1. Toxicity studies

1.1. *Haematococcus* algae.

1.1.1. Human safety study

In a recent clinical safety study with Aquasearch's ***Haematocccus pluvialis*** algal meal, 33 human volunteers (15 males and 18 females, age 28 to 62) ingested on a daily basis, for 29 consecutive days, either a Low Dose supplement containing 228 mg algal meal and 3.85 mg astaxanthin, or a High Dose supplement containing 1140 mg algal meal and 19.25 mg astaxanthin.[1]

Volunteers underwent a complete medical examination before, during and at the end of the study. The physician, examined specifically, but not exclusively, the weight, skin coloration, general appearance, blood pressure, vision and eye, (near and distant vision, color vision, depth perception, eye condition), ears and nose, mouth, throat and teeth, chest and lungs, and reflexes, for each volunteer.

This medical examination was complemented by extensive urine analyses and blood analyses (cell counts, hemoglobin, liver enzyme activity indicators, and other blood parameters) (Table 1). No ill effects or toxicity from ingestion of the supplement were

observed, confirming the absence of toxicity of Aquasearch's ***Haematococcus pluvialis*** algal meal.

1.1.2. Rat toxicity studies

Absence of toxicity of ***Haematococcus pluvialis*** has also been demonstrated in rats and mice, widely accepted animal models for safety assessment of human dietary supplements.

A 28-day sub-acute rat toxicity study, with ***Haematococcus pluvialis*** algal meal produced with Aquasearch's proprietary technology, failed to find any sign of toxicity of this algal meal.[2]Three groups of 20 rats each (10 males/10 females) were fed daily by gavage 0, 5, or 50 mg/kg algal meal in a corn oil suspension for 28 consecutive days (corresponding to daily doses of 0, 350 mg and 3,500 mg algal for 70-kg body weight). After sacrifice, the post-mortem observations, hematology and clinical chemistry failed to detect any sign of toxicity.

An earlier 13-day rat toxicity study demonstrated that the LD50 acute toxicity of ***Haematococcus pluvialis*** algal meal in rats was greater than 5000 mg/kg.[3] In this study, three separate groups of 10 rats (5 males and 5 females per group) were fed 5,000 mg/kg algal meal suspended in a 0.5% methylcellulose solution. Mortality, body weights, necropsy examination and pharmacotoxic signs were evaluated on each group. The study found no remarkable differences in body weights or visible abnormalities. The post-mortem examination after sacrificing the animals at the end of the study revealed no abnormalities.

Another acute toxicity trial was reported with male and female mice.[4] In this study, ***Haematococcus pluvialis*** algal meal was suspended in distilled water for gavage to give a 30% solution (w/v). The solution was given in a single dose, at dosages ranging from 10,417 to 18,000 mg/kg. No mortalities occurred and no abnormalities were observed in the post-mortem examination. When converted to a 70-kg body weight, these doses are equivalent to single doses ranging from 729 g to 1,260 g.

1.1.3. Other studies

In salmonids, numerous experiments have shown that ***Haematococcus pluvialis*** can be incorporated in the diet at dosages ranging from 0.1% to 6% without any negative effect on growth or survival.[5,6,7,8] A recent report showed no indication of disease, toxicity or neoplasia in fish fed ***Haematococcus pluvialis*** as a dietary source of astaxanthin.[4] The fish were reported in excellent nutritional status with abundant body fat. Studies have also

indicated that feeding ***Haematococcus pluvialis*** can enhance growth and/or survival in trout and shrimp.[8-10]

1.2. Astaxanthin.

Astaxanthin naturally appears in the human diet when seafood such as salmon, red fishes, shrimp, krill or lobster are eaten.

1.2.1. Human studies

The recent clinical safety study, mentioned above, proved the safety of astaxanthin from Aquasearch's ***Haematocccus pluvialis*** algal meal. [1] In that study, 33 human volunteers (15 males and 18 females, age 28 to 62) ingested on a daily basis, for 29 consecutive days, either 3.85 mg or 19.25 mg algal astaxanthin. As mentioned earlier, extensive blood and urine analyses were conducted throughout the study (Table 1), and the physician conducted a detailed medical examination. Based on the results of these urine and blood analyses and the observations of the physician, no sign of toxicity from astaxanthin was detected even at the higher dose.

In a study with healthy human patients, who ingested up to 14.4 mg/day astaxanthin for two weeks, no ill effect was reported.[11] On the contrary, a positive antioxidant effect of astaxanthin on serum Low Density Lipoprotein (LDL) was observed. In that study, thirteen healthy patients were selected, subdivided into 3 groups, and given three levels of astaxanthin daily, for two weeks, as follows: 5 patients fed 3.6 mg/day, 5 patients fed 7.2 mg/day, and 3 patients fed 14.4 mg/day. The astaxanthin was administered sublingualy in the form of a softgel capsule. Blood samples were taken and the LDL fraction was collected and exposed to an oxidizing agent. The study demonstrated that increasing doses of astaxanthin significantly and increasingly slowed down the oxidation of the LDL fraction.

1.2.2. Rat toxicity studies

In the recent study with Aquasearch's ***Haematococcus pluvialis*** algal meal, described above, rats ingested daily up to 1.15 mg astaxanthin per kg body weight (equivalent to 80.5 mg for 70-kg body weight per day), for 28 days, without showing any sub-acute toxicity sign.

Other animal studies on the effects of astaxanthin have shown that even higher doses could be fed to rats for prolonged periods. Some of these studies have demonstrated beneficial

results. In one study, feeding rats 500 ppm astaxanthin for 34 consecutive weeks resulted in reduced cancer occurrence in the intestinal and oral mucosa and improved the condition of the oral cavity.[12,13]

1.2.3. Safety of astaxanthin in food salmon – safe daily dose of astaxanthin

For years, astaxanthin has been added to aquaculture diets at levels of up to 200 mg/kg, without any toxic effect on target animals. Additionally, numerous studies have demonstrated improved growth, survival and immune response in fish and shrimp.[8-10,14-23] Astaxanthin is regularly added at 50 ppm or higher to commercial diets fed to food fish for prolonged periods, i. e., for up to 2 years in the case of farmed salmon.

According to the Code of Federal Regulations, astaxanthin is Generally Recognized As Safe ("GRAS") when used as a color additive in salmon foods, with a maximum inclusion of 80 mg/kg feed.[24] Numerous studies have shown that such an inclusion level results in accumulation of astaxanthin in the flesh of Atlantic salmon at levels between 4 and 10 mg/kg, and at even higher levels in other species (Table 2).

These levels in Atlantic salmon are comparable to or slightly higher than levels observed in their wild counterparts, but lower than levels found in other wild salmon species found on the Pacific coast of the United States, where values as high as 58 ppm in Sockeye salmon were reported by a recent FDA study.[25] (Average of astaxanthin measurements in this study were 13.8 ppm in Coho salmon and 40.4 ppm in Sockeye salmon).

It was noted that the main astaxanthin stereo isomer identified by the FDA researchers in the 5 species of wild Pacific salmon they studied, was the 3S,3'S stereo isomer, identical to that found in ***Haematococcus pluvialis***.[8,25]

Salmon, a fish rich in omega-3 fatty acids, is considered a healthy food, and, like other sources of these poly-unsaturated fatty acids, is highly recommended by nutritionists.[26-29] According to an epidemiological study on Alaska's native and non-native residents, the lowest rate of ischaemic heart disease mortality, less than one-third that of US Caucasians, occurred in Alaskan Eskimos who lived in an area with documented patterns of high salmon consumption by individuals with high blood concentrations of omega-3 fatty acids.[28] Based on the salmon flesh astaxanthin values mentioned above, a daily consumption of a 200-g portion of wild Sockeye salmon with 40 ppm astaxanthin in the flesh would lead to a daily ingestion of 8 mg astaxanthin per day. From a different point of view, the intake of a 5 mg supplement of astaxanthin corresponds to eating 500 g per day

of farmed rainbow trout or Atlantic salmon, 125 g of wild Sockeye salmon, or less than 100 g of krill.

Based on these published data, as well as the animal toxicity data publicly available, it may be inferred that the ingestion of 5 mg astaxanthin per day by an adult human is reasonably safe. This was further substantiated by Aquasearch's 29-day human safety study, which investigated the safety of 3.8 mg astaxanthin/day and 19 mg/day astaxanthin from ***Haematococcus pluvialis*** algal meal, i. e., almost four-fold higher than the assumed safe daily dose of 5 mg.[1]

The results of the extensive blood and urine analyses and complete physical examinations before, during, and at the end of the trial period, raised no apparent safety concern. The data were reviewed by two independent physicians, a clinical pathologist and a professional pharmacotoxicologist, all of who concurred that both doses were safe.

2. Non-mutagenicity of *Haematococcus*

A recent study[30] reported no mutagenic effect of ***Haematococcus pluvialis*** algae, using a mutagenicity test with *Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537, TA1538, and E.coli WP2 uvr A.

In this experiment, ***Haematococcus pluvialis*** algal meal was formulated in a 50mg/mL solution of dimethyl sulfoxide. The formulation was spread onto petri dishes in the presence of the microbial cultures with positive controls. The positive controls (mutagenic agents): 2-(2-furyl)-3-5(5-nitro-2-furyl)acrylamide, 1-ethyl-2-nitro-3-nitrosoguanidine, 9-aminoacridine, 2-aminoanthracene, and 2-nitrofluorene, showed a remarkable increase in the number of reverent colonies in every case, compared to the solvent control.

3. Carcinogenicity

Haematococcus pluvialis is not known to have any carcinogenic effect, or contain significant levels of recognized carcinogens. On the contrary, ***Haematococcus pluvialis*** contains a high level of astaxanthin which has widely demonstrated anticarcinogenic effects.[31-35]

4. Heavy metals

Haematococcus pluvialis algae produced and processed by Aquasearch for human food consumption meet the Federal Food and drug Administration's list of maximum tolerances:

- Heavy metals (as lead): < 10.0 ppm
- Mercury < 1.0 ppm
- Cadmium < 0.5 ppm
- Arsenic < 2.0 ppm
- Lead < 5.0 ppm

This has been confirmed by analyses of various batches (Lot HP980051[36] and Lot 990610Mix[37], a blend resulting from combining five batches: Lots 990513A, 990518B, 990520A, 990524A, 990526A, and therefore, highly representative of the quality of ***Haematococcus pluvialis*** algal meal produced with Aquasearch's technology).

5. Bacteriology

Manufacturing process follows FDA GMP recommendations for food supplements to avoid spoilage and contamination of ***Haematococcus pluvialis*** algal meal by harmful micro-organisms or other types of contaminants.

During the processing, the algal biomass is mechanically cell-broken to ensure a thorough rupture of cell walls, undergoes a pasteurization process, and is dried to a moisture content less than 5%. The pasteurization treatment ensures that the following bacteriological specifications in the final product are achieved, as confirmed by analyses by an independent laboratory[37]:

- Total aerobic plate count <1,000 CFU
- Total coliforms <10/g
- ***E. coli*** <10/g
- ***Salmonella*** absence in 25 g

6. Other natural toxic compounds and toxicity risks

Aquasearch is not aware of any significant or detectable levels of known carcinogenic or toxic compounds in ***Haematococcus pluvialis*** algae that could have a negative effect on human health.

Analyses on the algae meal have demonstrated absence of mycotoxins, and especially of aflatoxins.[36,37]

Haematococcus pluvialis may contain small amounts of canthaxanthin, a carotenoid

pigment closely related to astaxanthin. Analyses have shown that canthaxanthin concentrations in ***Haematococcus pluvialis*** algal meal produced with Aquasearch proprietary technology are less than 2% of total astaxanthin concentration. Aquasearch's proprietary technology maximizes astaxanthin biosynthesis by Haematococcus pluvialis and in so doing also minimizes the relative proportion of other carotenoids (including canthaxanthin).

At the levels of canthaxanthin encountered in Aquasearch's algal meal, a daily dose of 5 mg algal astaxanthin as a supplement would entail also ingesting 0.1 mg canthaxanthin per day. Although canthaxanthin has been demonstrated to have positive metabolic effects such as an anticancer activity,[38] there has been reports that, at high doses for prolonged periods, it can have negative effects. One case of aplastic anemia associated with canthaxanthin ingested for tanning purposes, was reported a few years ago[39]. Others have reported the appearance of crystalline formations in the retina of some individuals who ingested up to 66 g cantaxanthin over 24 months (corresponding to an average daily ingestion of 90 mg cantaxanthin per day) for tanning purposes[40]. However, later it was demonstrated that these canthaxanthin deposits in the retina could be reversed[39] In any case, the levels of canthaxanthin that would be ingested through a 5 mg astaxanthin dietary supplement formulated with Aquasearch's algal meal are nearly 1000-fold lower than the doses which were observed to cause canthaxanthin maculopathy. Therefore, they should represent no safety risk.

The rat toxicity and human studies which were conducted with Aquasearch's algal meal confirmed this. It should also be noted that FDA has approved canthaxanthin as a color additive in fish foods (up to 80 mg/kg feed, which can result in canthaxanthin deposition levels of 4 to 12 mg/kg fillet) and broiler diets, as well as in foods and drugs.[41] In foods, the limit authorized by FDA is 30 mg canthaxanthin per pound of solid food. The ingestion of 0.1 mg cantaxanthin in a dietary supplement containing 5 mg astaxanthin, is therefore well below the levels that would be encountered in foods that are considered safe by FDA.

7. Product specifications

A detailed description of the manufacturing process and of the specifications of ***Haematococcus pluvialis*** for use in dietary supplements are reviewed in a separate technical report.[42]

8. Metabolic effects of astaxanthin

Astaxanthin is a powerful natural antioxidant. There is a growing amount of scientific

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evidence not only on the safety of astaxanthin for human consumption, but on the positive metabolic effects that it may have. These findings have been reviewed in detail in Aquasearch Technical Reports TR.3002.001[43] and TR.3003.001 [44].

9. Dietary studies - safe daily dose of algal astaxanthin

Astaxanthin appears to be absorbed in the blood in the same way as other carotenoids. Carotenoids are absorbed by passive diffusion through the intestinal mucosa after being emulsified and solubilized in lipid micelles which are incorporated into chylomicrons when exiting the intestinal mucosal cells.[45] They are transported in the blood after being transferred from the chylomicrons to lipoproteins.

In a recent human study, a single dose of 100 mg dietary astaxanthin was not found to have any negative effect and demonstrated that astaxanthin has a similar absorption pattern to other carotenoids.[46] Astaxanthin was measured in the blood plasma of 3 middle-aged male subjects after ingestion of a single dose of 100 mg astaxanthin. Astaxanthin was readily absorbed and transported by various lipoproteins: chylomicrons/Very Low Density Lipoproteins, High Density Lipoproteins and Low Density Lipoproteins.

Plasma levels of astaxanthin peaked at 1.2 mg/L (= 2 µmol/L) after 6 hours and progressively declined over the next 66 hours to a 0.2 mg/L level. These levels and duration are comparable to levels reported in the literature for other carotenoids.[47-49] Astaxanthin appears to be absorbed at a similar rate than beta-carotene which peaks in the serum after 6 to 9 h.[49] In mice, astaxanthin also appeared to be absorbed quite effectively, when compared to beta-carotene or lutein.[49]

The official recommended dietary intake for vitamin A is 1,000 retinol equivalents, for men, and 800 for women.[51] This corresponds to 6 µg (***micro***grams) beta-carotene or 12 µg of other pro-vitamin A carotenoids.[51] On the other hand, practical levels of carotenoid intake are significantly higher. Epidemiological studies in North Europe have found daily ingestion of carotenoids ranging from 2.9 to 7.6 mg/ (***milli***grams) per day,[52-54] while in the US, the level of carotenoids supplied by the "normal" diet is estimated to be 1.5 mg beta-carotene per day.[51]

The Alliance for Aging Research, a US Citizen Advocacy organization for research to improve the health and independence of older people, has recommended 10 to 30 mg beta-carotene per day for optimal health, and doses of 20 to 180 mg beta-carotene for

many years have been used to treat erythropoietic protoporphyria, with no evidence of toxicity and without development of abnormally-elevated blood vitamin A levels.[51] In addition it should be noted that astaxanthin, unlike other carotenoids such as beta-carotene, has no provitamin A activity;[55,56] therefore it represents a lower risk of hyper-vitaminosis A.

It may be argued that because astaxanthin is closely related to canthaxanthin it could also have similar toxic effects as those described above. However, the available data indicate that astaxanthin consumption at no greater than the recommended dose of 5 mg per day poses no safety risk:

- The proposed daily intake of astaxanthin (5 mg) is much lower than the levels of canthaxanthin which were found to have toxic effects (up to 90 mg average daily intake for 24 months).

- The human safety study conducted with Aquasearch's algal astaxanthin found no changes in vision or eye condition in the patients. Another good indicator, skin coloration, did not change throughout the Aquasearch safety study.

- The post-mortem examination of the animals in Aquasearch's rat toxicity study also failed to find any adverse effect of astaxanthin supplementation at the doses tested.

- Researchers at the University of Illinois also reported that, in an animal model (rats), astaxanthin, unlike canthaxanthin, did not form crystalline depositions in the eye.[57] Furthermore, they demonstrated that astaxanthin can have a beneficial role in the protection of the eyes from UV-light damage.

In conclusion, based on published studies (reviewed above), on natural levels of astaxanthin found in seafood, and on the results of the studies conducted by Aquasearch, it appears that the consumption by a healthy adult human of a daily dose of 5 mg astaxanthin, in the form of a supplement formulated with 250 mg (or less) ***Haematococcus pluvialis*** algal meal produced with Aquasearch's proprietary technology, represents no safety risk. This suggested dose is approximately four times lower than the high dose which was demonstrated to be safe by Aquasearch's safety study.

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Table 1: List of analyses in human safety study conducted on Aquasearch's *Haematococcus pluvialis* algal meal.[1]

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Blood chemistry analyses

Serum glutamate pyruvate transaminase (SGPT)
Lactate dehydrogenase (LDH)
Glucose
Total protein
Total bilirubin
Blood urea nitrogen (BUN)
Creatinine
Total cholesterol
High-density lipoprotein (HDL) cholesterol
Triglycerides
Low-density lipoprotein (LDL) cholesterol (calculated)
Albumin
Globulin

Complete blood count (CBC)

White blood count (WBC)
Red blood count (RBC)
Hemoglobine (HGB)
Hematocrit (HCT)
Mean corpuscular volume (MCV)
Mean corpuscular hemoglobin (MCH)
Mean corpuscular hemoglobin concentration (MCHC)
Red cell distribution width (RDW)
Platelet count
Neutrophil (segs)
Lymphocytes
Monocytes
Eosinophils
Bsophils
Red blood cell morphology
Coagulation test (activated partial thromboplastin time, PTT)

Urinalysis tests

Color	pH
Appearance	Protein
Specific gravity	Glucose
Leukocyte esterase	Ketones
Nitrite	Urobilinogen
Blood	Bilirubin

Table 2. Levels of of astaxanthin in selected types of seafoods[8]

Seafood type	Astaxanthin Content (mg/kg)	Free/esterified	Main isomer
Sockeye salmon	26-37	Free,esterified**	3***S***,3'***S***
Coho salmon	9-21	Free,esterified**	3***S***,3'***S***
Chum salmon	3-8	Free,esterified**	3***S***,3'***S***
Chinook salmon	8-9	Free,esterified**	3***S***,3'***S***
Pink salmon	4-6	Free,esterified**	3***S***,3'***S***
Atlantic salmon	3-11	Free,esterified**	3***S***,3'***S***
Rainbow trout	1-3	Free,esterified**	3***S***,3'***S***
salmon eggs	0-14	Esterified***	N.A.
Red seabream	2-14	Esterified***	N.A.
Red seabream eggs	3-8	N.A.	N.A.
Peneaus monodon	10-150	Esterified,free**	3***S***,3'***S***
Lobster		Esterified,free**	N.A.*
Krill	46-130	Esterified***	3***R***,3'***R***
Krill oil	727	Esterified***	3***R***,3'***R***
Crayfish meal	137	Esterified***	N.A.*
Artic shrimp	1160	Esterified***	3***S***,3'***S***
Haematococcus pluvialis	10,000-30,000	Esterified***	3***S***,3'***S***

* Most crustaceans studied appear to have mostly the 3*S*,3'*S* form, unlike Krill.

** depending on tissues, free or esterified astaxanthin may be found

*** also contain a small proportion of free astaxanthin

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Return to Astaxanthin and Health
Return to Safety Information
Return to Clinical Safety Study

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KOREA FOOD & DRUG ADMINISTRATION

SUBJECT : REGARDING KRILL PRODUCT

QUESTION FROM SAM OH

: THERE IS EPA AND/OR DHA FOOD IN HEALTH FOOD CATERGORIES AND, ITS DEFINITION IS 'A THINGS FROM EDIABLE FISHES, AQUATIC ANIMALS AND ALGAE;

WE HAVE A KRILL OIL (OMEGA 3 FATTY ACIDS (EPA/DHA) CONTENT 40% UP) AND, WE WOULD LIKE TO KNOW WHETHER THIS KRILL OIL IS EPA AND/ OR DHA FOOD PLEASE INVESTIGATE AND LET US KNOW YOUR RESULT.

ANSWER FROM FOOD EVALUATION DEPARTMENT

: KRILL PRODUCT IS ACCEPTABLE AS A RAW MATERIAL FOR HEALTH FOOD BECAUSE IT IS CLASSIFIED INTO EDIABLE MATERIAL AND, KRILL OIL IS ALSO ACCEPTABLE AS EPA/DHA FOOD YOU CAN MEET THE KOREA FOOD CODE.

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APPENDIX B

CERTIFICATE OF ANALYSIS

PRODUCT: Neptune Krill Oil TM (NKL TM)

DESCRIPTION	PER INTAKE OF ONE 1 g CAPSULE (S)	PER INTAKE OF THREE 1 g CAPSULE (S)
Total Omega - 3 (mg)	354,0	1062,0
EPA	183,0	549,0
DHA	95,8	287,4
OTA	3,7	11,1
Linoleic acid	11,7	35,1
Linolenic acid	5,7	17,1
Oleic acid	101,0	303,0
Stearic acid	6,5	19,5
Palmitic acid	155,0	465,0
Palmitoleic acid	46,7	140,1
Total Phospholipids (mg)	575,0	1725,0
Phosphatidylcholine	520,0	1560,0
Phosphatidylinositol	2,5	7,5
Phosphatidylethanolamine	34,0	102,0
Phosphatidylserine	12,0	36,0
Sphingomyelin	6,0	18,0
Vitamins (IU):		
Vitamin A	809	2427
Vitamin E	1,0	3,0
Pigments (micro-grams) :		
Canthaxanthin	389,3	1 167,90
Astaxanthin	168,7	506,1
Metals (mg) :		
Zinc	0,007	0,020
Sodium	3,9	11,6
Potassium	2,1	6,2
Proximate Analysis (mg) :		
Protein	61	183
Fat	793	2379
Cholesterol	13	39
Solvent (ppm)	0,1	0,3
Heavy Metal (mg)	0,01	0,03

APPENDIX C

CERTIFICATE OF ANALYSIS

PRODUCT : Neptune AQUATEIN™ (NKA™)

Recommended daily dose = 3 gelcaps

DESCRIPTION	PER INTAKE OF ONE 300 mg GELCAP(S) (units / 300 mg)	THREE 300 mg GELCAP(S) (units / 900 mg)
Total Omega - 3 (mg):		
EPA	0,5	1,4
DHA	0,4	1,3
Total Phospholipids (mg):	1,8	5,5
Vitamins:		
Vitamin A (IU)	0,8	2,5
Vitamin D (IU)	5,3	15,8
Vitamin E (IU)	0,05	0,14
Vitamin B2 (mg)	0,02	0,06
Niacin (mg)	0,2	0,6
Metals (mg):		
Calcium	5,9	17,6
Magnesium	2,5	7,5
Potassium	6,8	20,3
Sodium	2,1	6,2
Amino Acids (mg):		
Alanine	13,1	39,2
Arginine	19,7	59,2
Aspartic Acid	21,6	64,8
Cystine	3,8	11,3
Glutamic Acid	30,0	90,0
Glycine	12,8	38,5
Histidine	5,6	16,7
Isoleucine	11,0	32,9
Leucine	18,4	55,1
Lysine	21,5	64,4
Methionine	6,2	18,6
Ornithine	3,2	9,5
Phenylalanine	14,2	42,6
Proline	11,9	35,7
Serine	6,6	19,7
Taurine	5,3	15,8
Threonine	3,6	10,8
Tryptophan	12,4	37,1
Tyrosine	12,8	38,5
Valine		
Proximate Analysis (mg):		
Protein	245,1	735,3
Fat	3,3	9,9
Carbohydrates	0,9	2,7

APPENDIX D

CERTIFICATE OF ANALYSIS

PRODUCT : Neptune LyO-Krill™ (NLK™)

DESCRIPTION	Recommended daily dose = 3 gelcaps ONE 300 mg Gelcap(s) (units / 300 mg)	THREE 300 mg Gelcap(s) (units / 900 mg)
Total Omega - 3 (mg):		
EPA	4,8	14,5
DHA	2,6	7,8
Total Phospholipids (mg):	7,4	22,1
Vitamins:		
Vitamin A (IU)	24,7	74,2
Vitamin D (IU)	51,5	154,4
Vitamin E (IU)	0,12	0,36
Vitamin B2 (mg)	0,05	0,14
Niacin (mg)	0,4	1,2
Metals (mg):		
Calcium	7,3	21,8
Magnesium	3,3	9,9
Potassium	6,0	18,0
Sodium	1,8	5,4
Amino Acids (mg):		
Alanine	13,4	40,3
Arginine	18,2	54,7
Aspartic Acid	19,9	59,7
Cystine	3,5	10,5
Glutamic Acid	28,4	85,2
Glycine	13,5	40,5
Histidine	5,2	15,5
Isoleucine	10,1	30,2
Leucine	17,6	52,7
Lysine	20,9	62,7
Methionine	5,8	17,5
Ornithine	3,0	9,1
Phenylalanine	13,0	38,9
Proline	11,6	34,9
Serine	5,4	16,3
Taurine	6,1	18,2
Threonine	10,5	31,5
Tryptophan	3,0	9,1
Tyrosine	10,5	31,5
Valine	11,8	35,4
Proximate Analysis (mg):		
Protein	232,2	696,6
Fat	10,2	30,6
Carbohydrates	4,2	12,6

APPENDIX E

CERTIFICATE OF ANALYSIS

PRODUCT : Neptune KRILL ENZYME™ (NKE™)

RECOMMENDED DAILY DOSE = 3 GELCAPS

DESCRIPTION	PER INTAKE OF	
	ONE 300 mg	THREE 300 mg
	GELCAP(S)	
	(units / 300 mg)	(units / 900 mg)
Total Omega - 3 (mg):		
EPA	2,6	7,7
DHA	0,9	2,8
Total Phospholipids (mg):	4,5	13,5
Vitamins:		
Vitamin A (IU)	12,3	36,9
Vitamin D (IU)	27,5	82,5
Vitamin E (IU)	0,08	0,25
Vitamin B2 (mg)	0,03	0,10
Niacin (mg)	0,3	0,9
Metals (mg):		
Calcium	6,4	19,1
Magnesium	1,9	5,8
Potassium	2,9	8,7
Sodium	6,6	19,8
Amino Acids (mg):		
Alanine	13,3	39,8
Arginine	19,0	57,1
Aspartic Acid	20,8	62,3
Cystine	3,6	10,9
Glutamic Acid	29,2	87,5
Glycine	13,2	39,5
Histidine	5,4	16,1
Isoleucine	10,5	31,6
Leucine	18,0	54,0
Lysine	21,2	63,6
Methionine	6,0	18,1
Ornithine	3,1	9,3
Phenylalanine	13,6	40,8
Proline	11,8	35,3
Serine	6,0	18,1
Taurine	6,1	18,2
Threonine	11,1	33,3
Tryptophan	3,3	10,0
Tyrosine	11,5	34,4
Valine	12,3	37,0
Proximate Analysis (mg):		
Protein	245,1	735,4
Fat	4,1	12,2
Carbohydrates	1,0	3,0

APPENDIX F

Scientific Report

April 17th 2002

AKER877ITC00057984

RX-0135.0079

April 17th 2002

Dear colleague,

I would like to take advantage of this opportunity to present to you the updated Scientific Report of Neptune Technologies & Bioressources Inc. In the next few pages, you will find a brief description of the primary Neptune extraction process designated as "Neptune OceanExtract™" followed by an introduction to the three initial products, "Neptune Krill Oil™", "Neptune Aquatein™" and "Neptune LyO-Krill™". You will also find a summary of the medical research performed with Neptune Krill Oil™. If you require a more specific profile of our products, it will be a pleasure to send it to you within a short delay.

Should you have any questions or comments, please feel free to contact me at Neptune Technologies & Bioressources Inc. main office or via e-mail at tinas@neptunebiotech.com.

Sincerely,

Tina Sampalis MD, PhD.
Vice President, Research
Neptune Technologies & Bioressources Inc.
500 Saint-Martin Blvd. West, suite 550,
Laval (Québec) H7M 3Y2
Tel. (450) 972-6291 1-888-664-9166
Fax (450) 972-6351

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NEPTUNE OCEANEXTRACT™

During 1998–1999, Neptune Technologies & Bioressources Inc. developed, in experimental phase at University of Sherbrooke, an extraction process **(Neptune OceanExtract™)** of natural health products with high nutritional value from marine biomasses. Krill was the first biomass used with this new process followed by seal, whole fish, fish residue and others. Subsequently, the procedure on krill was brought to a pilot industrial level in collaboration with the CRIQ. Neptune recently announced the initiation of the first phase of its industrialization plan and production is scheduled to begin in June 2002 under GMP and GLP compliance.

PROCEDURE

Neptune OceanExtract™ is a cold extraction process, allowing the extraction of:

a) **Neptune Krill Oil™** : an oil rich in Omega-3, phospholipids and potent antioxidants,
b) **Neptune Aquatein™** : a protein concentrate containing all essential amino acids.

ADVANTAGES OF "NEPTUNE OCEANEXTRACT™"

- This cold extraction process allows the preservation of the biological activity of all krill components;
- Neptune OceanExtract™ process is effective in the destruction of bacteria, offering a secure product for human consumption;
- Lipid alterations are minimal;
- Achieves complete utilization of the biomass allowing a high yield and minimal waste;
- Offers significant stability of the final products without the use of additive antioxidants or preservatives;
- Produces non-oxidized (peroxide value = 0) high quality products, rich in essential nutrients, highly stable, bacterial free and thus, safe for human consumption with noteworthy health benefits.

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NEPTUNE PRODUCTS

NEPTUNE KRILL OIL™

Neptune Krill Oil™, a marine oil offering a unique and natural mixture of essential nutrients. It is characterized by its high content of polyunsaturated fatty acids such as Omega-3 and 9, potent antioxidants such as astaxanthin, canthaxanthin, vitamin A, vitamin E as well as a novel flavonoid and unique phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin while demonstrating a remarkable and naturally acquired stability without the addition of any preservatives or antioxidants.

NEPTUNE AQUATEIN™

Neptune Aquatein™ , the dry fraction (or residue) remaining after the extraction of Neptune Krill Oil™. This residue's main characteristic features are its high protein content, 20 common amino acids including all essential amino acids, active and stable enzymatic activity, traces of residual pigments, traces of polypeptides (more or less short chains of amino acids resulting from the protein's self-digestion), chitin and growth agent.

NEPTUNE LYO-KRILL™

Neptune LyO-Krill™, a marine biomass product with preserved bioactive ingredients in the form of natural antioxidants such as: vitamin A, vitamin E, vitamin B1, niacin as well as axtaxanthin and canthaxanthin. Combined with P.U.F.A's and naturally enriched with calcium, copper and phospholipids, LyO-Krill™ represents an exceptional multi-functional dietary supplement, rich in essential amino acids, Branch Chained Amino Acids, digestive enzymes and peptides.

NEPTUNE KRILL ENZYMES™

Neptune Krill Enzymes™, a new marine enzyme supplement with 80% protein that contains 20% amino acids including 10 essential and 17% of BCAA (Branch Chained Amino Acids: Leucine, Isoleucine, Valine). Natural powerful digestive enzymes like proteases, phosphatases and phosphohydrolases combined with peptides that have potent biological activity offer a natural health product that can facilitate digestion and healing in multiple burn and trauma patients.

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NEPTUNE KRILL OIL™

1. OMEGA-3 FATTY ACIDS

a) Omega-3 fatty acids

Neptune Krill Oil™ contains a high quantity of Omega-3 fatty acids (EPA / DHA). The distribution of these Omega-3 fatty acids is what distinguishes Neptune Krill Oil™ as unique among the family of Omega-3 oils.

Scientific evidence proves that:

- DHA and EPA play a key role and may be beneficially supplemented for depression, schizophrenia, diabetes, cancer, rheumatoid arthritis and atherosclerosis (1, 2, 3);
- Omega-3 fatty acids (EPA & DHA) have a beneficial effect on dysmenorrhoea in adolescents (4);
- The effects of Omega-3 fatty acids supplementation in obese people and patients with arterial disease is verified in relation to genetic variation.

b) Omega-3 : Omega-6 ratio

Neptune Krill Oil™ offers an ideal ratio of Omega-3 : Omega-6, significantly favouring the Omega-3 which is deficient in our everyday diet.

- This ratio inhibits the effects of excess Omega-6 fatty acids, especially arachidonic acid, which is associated with chronic disease (5);
- Neptune Krill Oil™ contains 0.00 – 0.41 grams of arachidonic acid per 100 grams of oil.

c) Omega-9 (oleic acid)

Scientific evidence suggests that:

- Oleic acid slows down the gastrointestinal transit for patients with short bowel disease (38).

2. Potent antioxidants

a) Vitamin A – all-trans retinol

Scientific evidence suggests that:

- Vitamin A can reverse cell and tissue changes during neoplastic transformation indicating a potential role in cancer prevention (6);
- Retinol, beta-carotene, along with other dietary carotenoids, function as antioxidants that can prevent cellular damage at all stages from aging to carcinogenesis by decreasing the levels of the free-radicals that cause DNA damage (7).

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b) Vitamin E – alpha-tocopherol

Scientific evidence suggests that:

- Vitamin E may help prevent or delay coronary heart disease by inhibiting LDL- cholesterol oxidation and thrombus formation (8);
- Antioxidants such as vitamin E help protect against the damaging effects of free radicals, which may contribute to the development of chronic disease such as cancer (9).

c) Astaxanthin - esterified 3R-3R/Canthaxanthin

Astaxanthin has been proven to be:

- Twice as effective as beta-carotene (and about 80 times more effective than vitamin E) in quenching singlet oxygen in chemical solution (13);
- 50% more effective than beta-carotene and zeaxanthin, in preventing fatty acid peroxidation in chemical solution (14);
- In a membrane model, astaxanthin was found to be more effective at scavenging peroxyl radicals than was beta-carotene (15).

d) Flavonoids

Scientific evidence has shown that:

- Flavonoid antioxidant activity is accepted as a scientific fact (16-19);
- Epidemiological, clinical, and laboratory research on flavonoids, demonstrate the use of flavonoids in the prevention and/or treatment of cardiovascular disease, cancer, inflammatory conditions, asthma, periodontal disease, liver disease, cataracts and macular degeneration (17,18);
- Until today, there has never been a flavonoid extracted from anything other than plant, vegetable, fruit or algae.

3. Phospholipids (PL)

	Phospholipids	**46 g/100g**
(PC)	Phosphatidylcholine	24.0
(PS)	Phosphatidylserine	TBA
(PI)	Phosphatidylinositol	4.8
(SM)	Sphingomyelin	TBA
(PE)	Phosphatidylethanolamine	8.9

Phospholipids available in the market are derived from plant, egg yolk or cows (brain or liver);
Scientific evidence suggests that:

- Soy-based PC contain linoleic acid and alpha-linolenic acid as fatty acids;
- While plant-based PC have some beneficial effects on the brain, their fatty acids profiles are not ideal since they are different than those in the human brain;(20)

According to Dr. Michael Schmidt, krill-based phospholipids can be regarded as the preferred phospholipids for supporting peak brain performance due to their high content of polyunsaturated Omega-3 (EPA/DHA) fatty acids (20).

4. High natural stability

a. **Peroxide value 0.00**
This is the classic test for measuring oxidation in fresh oils. A peroxide value over 2 is an indicator that the product has a high rancidity potential and could fail on the shelf.

b. **Oil stability index Peroxide value <0.1 for more than 50 hours at 97.8°C**
All oils and fats have a resistance to oxidation, which depends on the degree of saturation, natural or added antioxidants or prior abuse. Oxidation is slow until this resistance is overcome, at which point oxidation accelerates and becomes very rapid. The length of time before this rapid acceleration of oxidation is the measure of the resistance to oxidation and is commonly referred to as the "induction period" or "Oxidative Stability Index".

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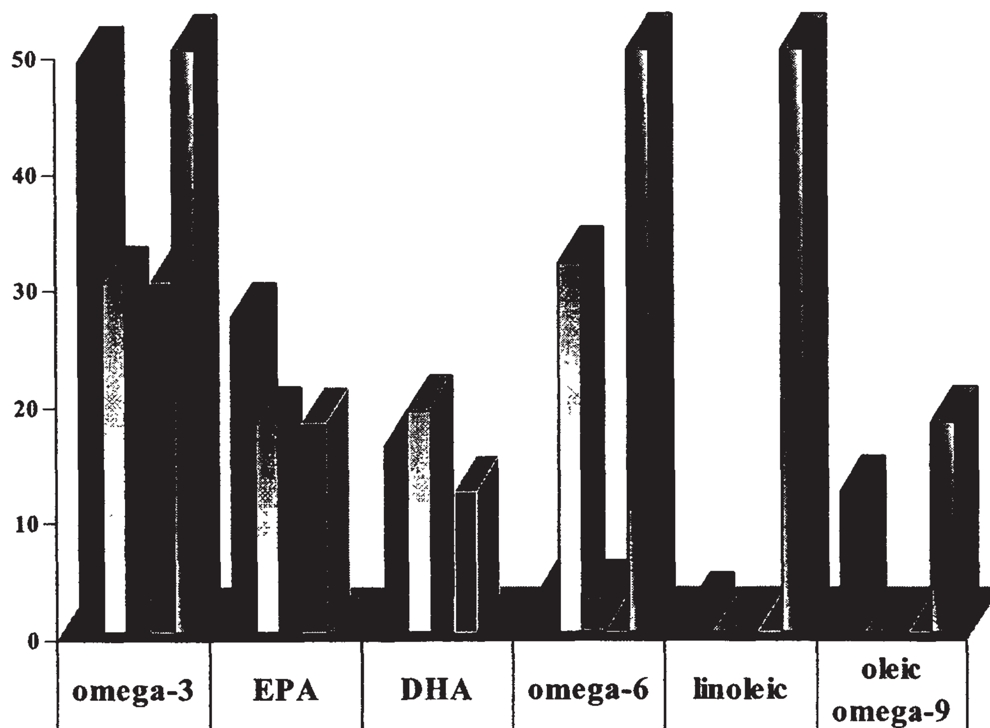
Preliminary results
Pilot tests
2002

Competition

Neptune Krill Oil™ vs. Other Oils
[g/100g]



Oils	omega-3	EPA	DHA	omega-6	linoleic	oleic omega-9
■ Krill	49	27	16	3	2	12
□ Fish	30	18	19	31,6	0	0
▨ Macademia	0	0	0	3	0	0
■ Cod Liver	30	18	12	0	0	0
■ Flax	56	0	0	70	70	18

Date of analysis: Feb. 08,2002

Fig. 1: Comparison of Neptune Krill Oil™ Omega fatty acids with other natural oils in the market.

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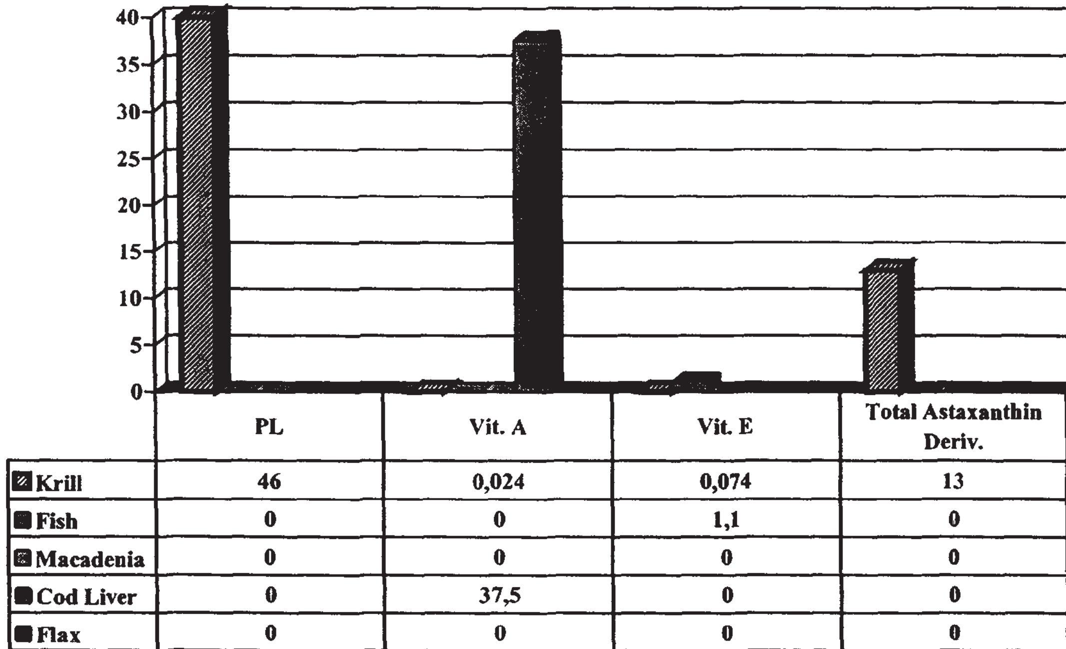
Preliminary results
Pilot tests
2002

Neptune Krill Oil™ vs. Other Oils
[g/100g]



	PL	Vit. A	Vit. E	Total Astaxanthin Deriv.
Krill	46	0,024	0,074	13
Fish	0	0	1,1	0
Macadenia	0	0	0	0
Cod Liver	0	37,5	0	0
Flax	0	0	0	0

Date of analysis: Feb. 08, 2002
Fig. 2: Comparison of Neptune Krill Oil™ phospholipids and antioxidants with other natural oils in the market.

The above histograms (Fig. 1 and 2):

- Demonstrate the advantages of Neptune Krill Oil™ versus other popular natural oils in the market;
- The alternative products and main competitors are fish oil, cod liver oil and flaxseed oil.
    - Fish oil contains a high quantity of Omega-3 and EPA / DHA in an excellent proportion but only a 1 : 1 Omega-3 : Omega-6 ratio. Furthermore, it does not contain antioxidants;
    - Cod liver oil has an excellent Omega-3 EPA / DHA content and very high vitamin A but does not contain phospholipids and offers none of the more potent antioxidants like astaxanthin and canthaxanthin;
    - Flaxseed oil contains a high quantity of alpha-linolenic acid, an Omega-3 fatty acid, but no EPA or DHA. In addition, the Omega-3:Omega-6 ratio is unfavourably reversed, in support of Omega-6.

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NEPTUNE AQUATEIN™

Neptune Aquatein™ , the dry fraction (or residue) remaining after the extraction of Neptune Krill Oil™. This residue's main characteristic feature is its high protein content of Antarctic Krill *Euphausia superba.* It offers the following genuine and potential attributes : high protein content, 20 common amino acids, all essential amino acids, active enzymatic activity, traces of residual pigments, traces of polypeptides (more or less short chains of amino acids resulting from the proteins self-digestion), chitin and traces of a growth agent.

PROTEINS

- Krill proteins are made of 20 common amino acids the contents of which are typically displayed (or described) in the manufacturers' specification sheets;
- The pure proteins value in Neptune Aquatein™ is 83%.

ENZYMES

- Scientific research has shown that Aquatein™ enzymes:
  - Are: lipases, phospholipases, alkaline phosphatase, acid phosphatase, esterase, trypsine, phosphohydrolase, â-glucoronidase, á-glucosidase, proteases, hyalurinases, and nucleases;
  - Have uncommonly high enzymatic activity;
  - React in low temperatures.

CHITIN

- The chitin and chitosan content of krill is, on a dry basis, between 2.4% to 2.7% and 2.8 respectively;
- Chitin is the source of a high-value added biopolymer chitosan with applications in the biomedical and pharmaceutical industries.

PEPTIDES

- Peptides correspond to pre-digested proteins, which, if taken raw, facilitate the digestion and assimilation;
- Peptides correspond to sections of proteins issued from the action of proteolytic enzymes contained in krill;
- These sections are chains of amino acids more or less short;
- The presence of noticeable contents of polypeptides adds nutrient value to the Neptune protein concentrate (Neptune Aquatein™).

NEPTUNE LYO-KRILL™

Neptune LyO-Krill™, a rich and well balanced source of essential nutrients: polyunsaturated fatty acids (P.U.F.A.), amino acids, phospholipids, vitamins and minerals. The superior quality of our product is attributed to the presence of intact absorbable, pre-digested proteins and biologically active polyunsaturated fatty acids, especially EPA and DHA and active enzymes. This condition ensures optimal biological action.

AMINO ACIDS, ENZYMES, PEPTIDES

- 20 amino acids, including all the essential amino acids, with 16% of BCAA (Branch Chained Amino Acids);
- The enzymes are: proteases, alkaline and acid phosphatases which are powerful, digestive and hydrolytic;
- Special peptides are present and have potent biological activity.

PHOSPHOLIPIDS

- Five phospholipids;
- May boost memory and improve concentration, learning, mood and overall well-being;
- May help to decrease the effects of neurodegenerative diseases (20).

OMEGA –3

- Omega-3 fatty acids are proven to have a significant beneficial effect on inflammatory and cardiovascular disease (1-4).

ASTAXANTHIN AND CANTHAXANTHIN

- Astaxanthin is recognized as one of the most potent natural antioxidant;
- Beneficial for cardiovascular diseases, primary cancer prevention, anti-aging, neurodegenerative diseases and ophthalmic disorders (13-15).

VITAMINS

- Vitamin A (all-trans retinol) , an essential fat-soluble vitamin with proven photoprotective and anti-aging potential (6,7).

RESEARCH

CARDIOVASCULAR DISEASE

- A recent study performed by Andrioli et al.(21) indicated that the systemic administration of fish oil rich in Omega-3 fatty acids inhibits platelet adhesion and thus plaque formation with an important determinant being the Omega-6 / Omega-3 ratio (17);
- Meydani showed that Omega-3 polyunsaturated fatty acids in fish oil have protective effects on cardiovascular disease by reducing the vascular endothelial inflammation in atherosclerosis;
- Postoperative daily administration of Omega-3 fatty acids in heart transplant recipients is effective as hypertension prophylaxis as proven by Guardia et. al.(18,21);
- Furthermore, astaxanthin has been shown in both *in vitro* experiments and in human subjects to be effective for the prevention of the oxidation of low density lipoprotein. Cos et al. (19).

The increased content of Omega-3 and antioxidants in Neptune Krill Oil™ allows us to presume beneficial effects for the maintenance of a healthy cardiovascular system.

Neptune is presently conducting a series of prospective randomized double blind trials in order to investigate the health benefits of Neptune Krill Oil™ on cardiovascular disease.

NEOPLASTIC DISEASE

- Retinols have been proven to have prophylactic effects against UV radiation induced skin cancer (6);
- Astaxanthin has been proven to be twice as effective as beta-carotene (and about 80 times more effective than vitamin E) in quenching singlet oxygen in chemical solution (13);
- 50% more effective than beta-carotene and zeaxanthin, in preventing fatty acids peroxidation in chemical solution (14);
- In a membrane model, astaxanthin was found to be more effective at scavenging peroxyl radicals than was beta-carotene (15,23).

The high content of all-trans retinols and astaxanthin in Neptune Krill Oil™ justifies further investigations of possible anticarcinogenic properties of our product preparation.

Neptune is conducting a study to evaluate the photoprotective potential of Neptune Krill Oil™ against UVB-induced skin cancer.

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RHEUMATOID ARTHRITIS

- Piet Geusens et al. showed that eicosanoids have an inhibitory effect on the formation of 2-series prostanoids and 4-series leukotrienes resulting in an improvement in the severity of symptoms and a decrease in the amount of corticosteroids and NSAIDS consumed (24);

- These observations are confirmed by Kremer et al., who demonstrated that Omega-3 fatty acids significantly inhibits the production of IL-1 (3).

Neptune is conducting a research study in order to evaluate the potential effects of Neptune Krill Oil™ supplementation on the clinical course of rheumatoid arthritis.

FACIAL WRINKLE REDUCTION

- The main property of retinol is the significant increase in cell differentiation. Based on their regenerative properties, retinols have been proven to be effective in the reduction of wrinkles when used topically (25, 26);

- Astaxanthin preparations have been shown to be efficient for the prevention of light aging of skin (23).

Neptune Krill Oil™ is a natural source of retinol and astaxanthin combined which could potentially increase their efficiency.

Neptune is conducting a research project in order to measure the effects of Neptune Krill Oil™ on aging and facial wrinkles.

TRANSDERMAL TRANSPORT

- Santoyo and Ygartua (28) demonstrated that percutaneous absorption can be enhanced with the topical use of fatty acids and phospholipids (24,25).

The high fatty acid and phospholipid composition and the significant all-trans retinol content with vitamin E in Neptune Krill Oil™ may facilitate the transdermal transportation of creams, ointments, gels or lotions.
Neptune is testing these properties on experimental models (nude mice) with skin analogous to that of human skin. The objectives of this study will be to evaluate the efficacy of Neptune Krill Oil™ as a substrate for topical treatments and to verify the speed of transdermal absorption of Neptune Krill Oil™ alone or as a substrate to other products in the cosmeceutical and/or biopharmaceutical industries.

Neptune Technologies & Bioressources Inc., recognizing the trend of modern medicine is investing in Research & Development of natural, efficient and pure products for the nutraceutical, biopharmaceutical and cosmeceutical industries.

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APPENDIX G

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RX-0135.0094

NEPTUNE KRILL OIL™
&
AQUATEINE™ (Krill Protein Concentrate)

EXTRACTION PROCEDURES

AKER877ITC00058000

RX-0135.0095

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
DUBUC, Jean, H.
Goudreau Gage Dubuc & Martineau
Walker
The Stock Exchange Tower
Suite 3400, P.O. Box 242
800 Place Victoria
Montreal, Quebec H4Z 1E9
CANADA

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year)	
27 April 2000 (27.04.00)	
Applicant's or agent's file reference: AML/10857.274	**IMPORTANT NOTICE**

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/CA99/00987	21 October 1999 (21.10.99)	21 October 1998 (21.10.98)
Applicant: UNIVERSITE DE SHERBROOKE et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,CN,JP,KP,KR,MA,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
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(54) Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

(57) Abstract

Provided herein is a method for extracting lipid fractions from marine and aquatic animal material by acetone extraction. The resulting non-soluble and particulate fraction is preferably subjected to an additional solvent extraction with an alcohol, preferably ethanol, isopropanol or *t*-butanol or an ester of acetic acid, preferably ethyl acetate to achieve extraction of the remaining soluble lipid fraction from the marine and aquatic animal material. The remaining non-soluble particulate contents is also recovered since it is enriched in proteins and contains a useful amount of active enzymes. Also provided herein is a krill extract.

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Fish farming

Among the lipids found in krill, *Calanus* and fish, high concentrations of fatty acids 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid) are present. These fatty acids are essential nutrients and are beneficial as fish feed. Furthermore, these essential nutrients are carried over in human diet by eating the fish grown on such diets.

Animal feed

Animal feed diets rich in omega-3 fatty acids may increase the level of unsaturated fatty acids and decrease cholesterol levels of meat. This property is already exploited in the poultry industry to improve the quality of eggs.

Various methods for extracting marine and aquatic animal oils are known. For example, it is known to extract fish oil using organic solvents such as hexane and ethanol. It is also known to measure the fat content in fish muscle tissue using solvents such as acetone.

USP 4,331,695 describes a method using pressurized solvents which are gaseous at room temperature, such as propane, butane or hexane. The extraction is performed at preferred temperatures of 15 to 80°C on shredded vegetable or finely divided animal products. The extracted oils are then made to precipitate under high pressure and elevated temperatures of 50 to 200°C. However, hexane is a poor extraction solvent for marine animals such as krill. Furthermore, the high temperatures used in the precipitation step negatively alters the lipids.

Canadian Patent Application 2,115,571 describes a method for extracting oils from various brown and read algae species. The method provides for example Soxhlet extraction using nearly pure ethanol for 40 hours.

USP 5,006,281 describes a method for extracting oil from marine and aquatic animals such as fish. The marine and aquatic animal is first treated with an antioxidant compound, finely divided and centrifuged to separate the oil phase from

the aqueous phase and solid phase. The oil phase is then further treated with antioxidant to remove undesirable odour or taste.

Canadian Patent 1,098,900 describes a method for extracting oils from krill. The
5 method involves emulsifying fresh or defrosted krill in an aqueous medium. The oil fraction is recovered by centrifugation.

Folch in the article published in the year 1957 in J. biol. Chem. 226: 497-509 *"A simple method for the isolation and purification of total lipids from animal tissues"*
10 proposes an extraction method using chloroform and methanol. This method is not commercially feasible because of the toxicity of the solvents involved.

However, prior art processes are generally commercially unfeasible or provide low quantitative yields. Thus, it is an object of the present invention to provide an
15 improved marine and aquatic animal oil extraction method allowing recovery of a valuable lipid fraction and separate recovery of a valuable protein rich solid residue that comprises active enzymes.

Other objects and further scope of applicability of the present invention will become
20 apparent from the detailed description given hereinafter. It should be understood, however, that this detailed description, while indicating preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art.

25

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Gas-liquid chromatography of fatty acids from dry krill (chloroform-methanol)

Figure 2. Gas-liquid chromatography of fatty acids from dry krill (acetone)

30 Figure 3. Gas-liquid chromatography of fatty acids from frozen krill (acetone)

Figure 4. Gas-liquid chromatography of fatty acids from frozen krill (ethanol)

Figure 5. Gas-liquid chromatography of fatty acids from frozen krill (*t*-butanol)

Figure 6. Gas-liquid chromatography of fatty acids from frozen krill (ethyl acetate)
Figure 7. Thin-layer chromatography of neutral lipids of *Calanus* sp. and *M. norvegica*
5 Figure 8. Thin-layer chromatography of neutral lipids of *E. pacifica*
Figure 9. Thin-layer chromatography of neutral lipids of *M. schmitti*
Figure 10. Thin-layer chromatography of neutral lipids of *G. galeus*
Figure 11. Thin-layer chromatography of neutral lipids of Angel Shark
Figure 12. Thin-layer chromatography of phospholipids of *Calanus* sp. and
10 *M. norvegica*
Figure 13. Thin-layer chromatography of phospholipids of *E. pacifica*
Figure 14. Thin-layer chromatography of phospholipids of *M. schmitti*
Figure 15. Thin-layer chromatography of phospholipids of *G. galeus*
Figure 16. Thin-layer chromatography of phospholipids of Angel Shark
15 Figure 17. Influence of the volume of acetone on lipid extraction (E. pacifica)
Figure 18. Influence of incubation time in acetone on lipid extraction (E. pacifica)
Figure 19. Influence of the volume of ethanol on lipid extraction (E. pacifica)
Figure 20. Influence of incubation time in ethanol on lipid extraction
20 (T. raschii)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Before describing the present invention in detail, it is to be understood that the invention is not limited in its application to the process details described herein. The
25 invention is capable of other embodiments and of being practised in various ways. It is also to be understood that the phraseology or terminology used herein is for the purpose of description and not limitation.

The method of the invention comprises suspending freshly collected marine and
30 aquatic material in acetone. Lipids are extracted with a ketone such as acetone. This allows a rapid dehydration of animal tissue and a migration of the lipid fraction to the solvent. The dry residue is a valuable product rich in active enzymes.

In a preferred embodiment, the extraction is carried out by successive acetone and alcohol treatments. Preferred alcohols are isopropanol, and *t*-butanol. The alcohol may also be substituted with an ester of acetic acid such as ethyl acetate. The procedure produces two successive lipid fractions and a dry residue enriched in protein, including active enzymes. Recovery of total lipids is comparable to the Folch et al. (1957) procedure reported in the background of the invention. It has been tested with krill, *Calanus*, fish and shark tissues.

Surprisingly, it was found that successive extraction treatments as proposed by the present invention has a better yield in lipid extraction that single solvent system extractions. The extraction using two successive solvents which starts with a ketone such as acetone is especially advantageous since the acetone, in effect, dehydrates the animal tissue. Having the animal tissue in dehydrated form greatly facilitates the extraction process with the second solvent, alcohol or an ester of acetic acid such as ethyl acetate.

In the case of zooplancton such as krill and *Calanus* and in the case of fish-filleting by-products such as fish viscera, it is noted that extraction with acetone alone may be sufficient to allow a cost-effective recovery of lipid fractions and separate recovery of a dry solid product rich in proteins including active enzymes.

The general extraction method of the present invention will now be described. The starting material consisting of freshly harvested and preferably finely divided marine and aquatic animal material is subjected to acetone extraction, for at about two hours and preferably overnight. However extraction time is not critical to the yield of lipid extraction. To facilitate extraction, it is preferable to use particles of less than 5mm in diameter. Extraction is preferably conducted under inert atmosphere and at a temperature in the order of about 5°C or less.

Preferably, the beginning of the extraction will be conducted under agitation for about 10 to 40 minutes, preferably 20 minutes. Although extraction time is not critical, it

was found that a 2 hour extraction with 6:1 volume ratio of acetone to marine and aquatic animal material is best.

The solubilized lipid fractions are separated from the solid material by standard
5 techniques including, for example, filtration, centrifugation or sedimentation. Filtration is preferably used.

After separation by filtration on an organic solvent resistant filter (metal, glass or paper) the residue is optionally washed with pure acetone, preferably two volumes
10 (original volume of material) to recover yet more lipids. The combined filtrates are evaporated under reduced pressure. Optionally, flash evaporation or spray drying may be used. The water residue obtained after evaporation is allowed to separate from the oil phase (fraction I) at low temperature.

15 The solid residue collected on the filter is suspended and extracted with alcohol, such as ethanol, isopropanol, *t*-butanol or alternatively with ethyl acetate, preferably two volumes (original volume of material). The filtrate is evaporated leaving a second fraction of lipids (identified as fraction II). Although the extraction period is not critical, it was found that an extraction time of about 30 minutes is sufficient at
20 temperatures below about 5°C.

Temperature of the organic solvents, except *t*-butanol, and temperature of the sample are not critical parameters, but it is preferable to be as cold as possible. However, in the case of *t*-butanol which is solid at room temperature, it is important
25 to warm it before using it and to perform the extraction at 25 °C immediately.

Comparative examples

To compare the efficiency of the extraction process, a classical technique (Folch et al. 1957) using chloroform and methanol was applied to krill. This method is the
30 reference for measuring efficiency of the extraction process. Another comparison has been made with a technique using hexane as the extraction solvent. Lipid recovery

was estimated by suspending lipid fractions in small volumes of their original solvents and measuring by gravimetry small aliquots after evaporation.

For all examples provided herein, the method of the present invention involving
5 acetone extraction followed by extraction with a second solvent (ethyl acetate, for example) gave a translucent oil having appearance and properties more attractive than any oil obtained by the classical technique of Folch et al. (1957).

To analyze lipid composition, 780 µg of each extract was loaded on silica-gel plates
10 and fractionated by thin layer chromatography, TLC (Bowyer et al. 1962) with the following solvents. Neutral lipids: hexane, ethyl ether, acetic acid (90:10:1, v/v) and phospholipids: chloroform, methanol, water (80:25:2, v/v). Fatty acid composition of *E. pacifica* was analyzed by gas liquid chromatography, GLC (Bowyer et al. 1962, see bibliography) including some modifications to the original technique: 2h at 65°C
15 instead of 1h at 80°C, three washes with hexane instead of two and no wash with water.

To get rid of traces of organic solvents, lipid fractions I and II are warmed to about 125°C for about 15 minutes under inert atmosphere.
20

Fat was analyzed according to the American Oil Chemist's Society (AOCS). The following criteria have been used to analyze the lipids extracted: saponification and Wijs iodine indexes and moisture-volatile matter levels. Cholesterol content has also been determined by the method of Plummer 1987 (see bibliography). The same
25 analyzes and others have been made by an independent laboratory under Professor Robert Ackman's supervision (Canadian Institute of Fisheries Technology, DalTech, Dalhousie University, Halifax, Nova Scotia, Canada). This includes Wijs iodine index, peroxide and anisidine values, lipid class composition, fatty acid composition, free fatty acid FAME, cholesterol, tocopherol, all-*trans* retinol, cholecalciferol,
30 asthaxanthin and canthaxantin contents.

Table 1 shows that higher levels of lipids are extracted from dry krill by acetone followed by ethanol as compared to the classical procedure of Folch et al. (1957).

Table 2 shows the results of lipid extraction from frozen *Euphausia pacifica*, a species of krill from Pacific Ocean. Assuming an eighty percent content of water, the lipid content is comparable to dry krill as shown in Table 1. Isopropanol, *t*-butanol and ethyl acetate, as solvent for the second extraction, give a yield less important than ethanol, but are not necessarily less effective in lipid recovery since ethanol carries more impurities than isopropanol, *t*-butanol or ethyl acetate. Then, they can be used as second solvent after acetone as well. Variations between results from acetone extractions are mainly due to the water-oil separations. These separations are influenced by the quantity of residual acetone in the water-oil solution after acetone evaporation. This quantity of acetone varies from an experiment to another, because the evaporation system used at a small scale is less reproducible (at the industrial scale, the evaporation step will be optimized). Single solvents have also been tested to extract the totality of lipids from krill. This shows that ethyl acetate (1,37% extraction rate), as hexane (0,23% extraction rate) are not good solvents, compared to acetone alone (1,86% extraction rate, and even greater extraction rates with an efficient acetone evaporation system).

One of the main advantages of the procedure is the removal of bacteria from extracts (lipid fraction and solid protein-rich material). Indeed, samples of *E. pacifica* incubated in different ratios of acetone at 4°C for 112 days have been inoculated on NA medium containing Bacto™ beef extract 0,3%, Bacto™ peptone 0,5% and Bacto™ agar 1,5% (Difco Laboratories, Detroit, USA) then incubated at room temperature or 4°C for 18 days. No significant bacterial growth was observed at a ratio of 1 volume of acetone per gram of krill. At higher proportions of acetone (2 volumes and 5 volumes), there was no bacterial growth at all, which means that acetone preserves krill samples. Acetone is known as an efficient bactericidal and viricidal agent (Goodman et al. 1980).

Table 3 shows the yield of lipids from *M. norvegica*. The percentage of lipids (3,67%) is comparable to the one obtained with *E. pacifica* (3,11%) shown in Table 2. Variations can be attributable to diet and time (season) of collection, which are different for those two species.

5

Table 4 shows the influence of grinding on the efficiency of extraction of *M. norvegica* lipids. These extractions were carried out under optimal conditions and show the definite advantage of the procedure over the classical method (4,46 % versus 3,30 %). It also shows that grinding may be an important factor when the

10 species is large (4,46% versus 3,53 %).

Table 5 reports on lipid extraction from *Calanus*. Considerable quantities of lipids were obtained. Some variations in *Calanus* species composition may explain the variations between experiments 1 and 2 (8,22 % and 10,90 % of fresh weight).

15

Tables 6-8 report the total amount of lipids extracted from fish tissue. The method of the present invention was demonstrated on mackerel, trout and herring. The method was demonstrated on peripheral tissues (mainly muscles) and viscera. Advantageously, the present method would permit the recovery of valuable lipid

20 fractions from parts of fish that are usually wasted after the withdrawal of fillets of the fish. Those fish tissues not used after the transformation of the fish for human consumption could be stored in acetone, and lipids extracted therefrom in accordance with the present invention even if the method Folch [1957] recovers more lipid than our method. Indeed small amounts of lipids from mackerel (0.52%

25 from viscera and 1,45% from tissues) have been extracted by the method of Folch after a first extraction with acetone and ethanol as described in the present invention. Comparative extractions with the method described in the present invention carried out in parallel with the method of Folch on trout and herring show superior recovery with the latter. However, it is noteworthy that the Folch method can not be applied

30 for the recovery of lipids for commercial uses (because of toxicity).

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In Tables 9 to 11, are shown results of lipids extraction from shark liver tissues. There is no marked difference in results between techniques within a species. Table 12 shows the fatty acid composition of krill oil (e. pacifica) following extraction in various solvents.

5

Tables 13 shows some characteristics features of fraction I (acetone) and fraction II (alcohol or ethyl acetate) for krill oil (e. pacifica). First, the saponification index of fraction I (130,6) indicates that this fraction contains fatty acids with longer chains, compared to fraction II (185,7). The Wijs iodine index of fraction I shows that this
10 fraction contains high levels of polyunsaturated fatty acids. As compared to olive oil which has an index of 81.1. It explains why fraction I is liquid at room temperature. It is well known that unsaturated fatty acids have a fusion point inferior to the one of their saturated homologues. The same observations are made for fraction II which has a iodine index of 127,2. The fatty acid composition shown in Table 12
15 corroborates these iodine indexes: fraction I has a high percentage (30,24%) of polyunsaturated fatty acids (pentaenes+hexaenes) and so fraction II (22,98%). Finally, Table 13 shows also that fraction I is comprised of 10,0% of volatile matter and humidity after evaporation of the solvent. For the same test, the fraction II gives a value of 6,8%. To get rid of traces of solvents, it is important to briefly heat (to
20 about 125°C, for about 15 min) the oil under nitrogen.

Results on krill oils obtained in accordance with the method of the present invention (fraction I extracted with acetone and fraction II extracted with ethyl acetate) are provided in Tables 13, 14, 15, 16, 17 and 18. It is noteworthy to mention that in
25 Table 18, the carotenoids content was significantly high as measured in terms of two carotenoids namely asthaxanthin and canthaxanthin. Indeed, duplicates analyzes revealed values of 92 to 124 µg/g of lipid fraction for asthaxanthin and 262 to 734 µg/g for canthaxanthin. Thus, for the purpose of the present invention it may be said that the krill extract comprises asthaxanthin at least 75 and preferably at least 90
30 ug/g of lipid fraction. In the case of canthaxanthin, at least 250 and preferably at least 270 µg/g of lipid fraction. Low values for peroxide and anisidine are advantageous and are due to the presence of high levels of natural antioxidants

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(astaxanthin and canthaxanthin). These compounds are indicative of favourable pharmaceutical or cosmetological properties of the krill extract whereby high levels of carotenoids indicate excellent transdermal migration characteristics. Thus, krill extract is a good candidative for transdermal delivery of medicines.

5

Table 19 shows the best mode of the method in accordance with the present invention for lipid extraction of aquatic animal tissues.

Table 20 shows that the enzyme activity of the solid fraction is maintained following

10 the method of the present invention. Indeed, the demonstration was completed for solid krill residue obtained after successive acetone and ethyl acetate extraction. Proteolytic activities were measure by the liberation of amino groups by spectrophotometric assay using *o*-pthaldialdehyde as reagent. Protein concentrations were measured by the Bradford method. Soluble proteins were

15 extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration. At the end of incubation at 37°C in 50mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH_3 group was measured in the supernatant according to the method of Church et al. [1983, J Dairy Sci 66: 1219-1227].

20

Figures 1 to 6 show chromatograms of fatty acid composition of *E. pacifica* lipids. On each of them, high proportions of 20:5 and 22:6 fatty acids (characteristic of marine and aquatic oils) are noticeable and represented by two distinct peaks. Data are shown in Table 12.

25

Variations in lipid patterns of neutral lipids (from Figure 7 to Figure 11) from one species to another are attributable to the differences in food sources. Within a species (*E. pacifica*, for example) there is no marked variation between lipid patterns obtained from different techniques of lipid extraction. Concerning phospholipids

30 (Figure 12 to Figure 16), the opposite is observed: variations are explained by the different extraction processes of lipids since the same species do not lead to the same lipid pattern. Lipids from shark species (extracted by the mentioned methods)

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and commercial cod-liver oil (sample available from Uniprix drugstores, Province of Québec, Canada) are mainly composed of neutral lipids as opposed to phospholipids.

5 The influence of the volume of solvent and incubation time on the efficiency of the acetone to extract lipids from *E. pacifica* is illustrated in Figures 17 and 18, respectively. A ratio of 1:6 (w/v) produced optimal yield with near complete extraction after 2h. The second extraction step has been experimented with ethanol. The volume of this solvent does not appear to be critical since the same yield was 10 obtained with different volumes of ethanol (Figure 19), but incubations time in ethanol should be at least 30 minutes as indicated by the results on Figure 20.

One of the inventors, Dr. Adrien Beaudoin, has ingested the different lipid fractions of krill. No side effect profile was observed.

15

Although the invention has been described above with respect with one specific form, it will be evident to a person skilled in the art that it may be modified and refined in various ways. It is therefore wished to have it understood that the present invention should not be limited in scope, except by the terms of the following claims.

20

Demonstration that krill residue, obtained after acetone and ethyl acetate extraction, contains enzyme proteolytic activities. Proteolytic activities were measured by the liberation of amino groups by spectrophotometric assay using *o*-phthaldialdehyde as reagent. Protein concentrations were measured by the Bradford method.

25

The enzyme source was the residue obtained after acetone and ethyl acetate extractions of lipids. Soluble proteins were extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration.

30 At the end of incubation at 37°C in 50 mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH_3 groups were measured in the supernatant according to Church and al. 1983.

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10

TABLE 1. EXTRACTION OF DRY KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	8,00		
	ethanol [b]	7,60	**15,60**	
2-	"	19,70		
		6,90	**26,60**	
3-	"	8,15		
		11,20	**19,35**	
4-	"	6,80		
		13,60	**20,40**	
				20,49±3,95
5-	chlor : MeOH [c]		**15,50**	
6-	"		**14,90**	
				15,20±0,30

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), no incubation.
[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 night at 4°C, following a first extraction with acetone.
[c] :Folch et al. 1957.

TABLE 2. EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	1,17		
	ethanol [b]	1,23	**2,40**	
2-	"	3,05		
		1,09	**4,14**	
3-	"	1,53		
		1,26	**2,79**	
				3,11±0,91
4-	acetone [a]	2,45		
	isopropanol [b]	0,70	**3,15**	
5-	"	1,80		
		0,80	**2,60**	
6-	"	1,60		
		0,80	**2,40**	
				2,72±0,39

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TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
7-	acetone [a)] *t*-butanol [c)]	2,15 0,47	**2,62**	
8-	"	2,11 0,40	**2,51**	
9-	"	2,37 0,45	**2,82**	**2,65±0,16**
10-	acetone [a)] ethyl acetate [b)]	2,28 0,21	**2,49**	
11-	"	1,09 0,16	**1,25**	
12-	"	2,54 0,09	**2,63**	**2,12±0,76**
13-	combined acetone-ethanol [d)]		**3,28**	
14-	"		**3,02**	
15-	"		**3,25**	**3,18±0,14**
16-	ethyl acetate [e)]		**1,32**	
17-	"		**1,49**	
18-	"		**1,31**	**1,37±0,10**
19-	hexane [e)]		**0,31**	
20-	"		**0,18**	
21-	"		**0,20**	**0,23±0,07**
22-	chlor:MeOH [f)]		**2,37**	

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TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
23-	"		2,07	
24-	"		2,62	
				2,35±0,28

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:6 (w/v), incubated 2 h at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[c] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 25°C, following a first extraction with acetone.
[d] :Extraction made with a sample-acetone-ethanol ratio of 1:5:5 (w/v/v), incubated 2 h at 4°C.
[e] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2 h at 4°C.
[f] : Folch et al. 1957.

TABLE 3. EXTRACTION OF FROZEN KRILL LIPIDS(M. norvegica)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	1,82		
	ethanol [b]	1,82	3,64	
2-	"	1,15		
		2,35	3,50	
3-	"	1,68		
		2,19	3,87	
				3,67±0,15

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

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TABLE 4. INFLUENCE OF GRINDING ON EXTRACTION OF FROZEN KRILL LIPIDS (M. norvegica)

Exp. No.	Technique	Krill ground before 1st extraction	Yield (%)	Total (%)
1-	acetone [a]	yes	3,10	
	ethanol [b]		1,07	4,17
2-	"	no	2,14	
			1,39	3,53
3-	"	yes	3,32	
			1,14	4,46
4-	chlor : MeOH [c]	yes		3,30
5-	"	yes		3,26

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:6, incubated 2 h at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:2, incubated 30 min at 4°C, following a first extraction with acetone.
[c] :Folch et al. 1957.

TABLE 5. EXTRACTION OF FROZEN *Calanus* LIPIDS (*Calanus* sp.)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	6,18		
	ethanol [b]	2,04	**8,22**	
2-	"	8,64		
		2,26	**10,90**	
				9,56±1,34

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

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TABLE 6. EXTRACTION OF FRESH FISH LIPIDS (Mackerel)

Exp. No.	Technique	Yield (%)	Total (%)
1- viscera	acetone [a)]	6,11	
fish 1	ethanol [b)]	0,59	**6,70**
2- tissues	"	3,78	
fish 1		0,91	**4,69**
3- viscera	"	10,46	
fish 2		0,57	**11,03**
4- issues	"	6,65	
fish 2		1,41	**8,06**
5- viscera	"	8,39	
fish 3		0,66	**9,05**
6- tissues	"	5,27	
fish 3		0,97	**6,24**
7- viscera	"	8,47	
fish 4		0,69	**9,16**
8- tissues	"	8,40	
fish 4		1,02	**9,42**
9- viscera	chlor:MeOH [c)]		**0,52**
fish 1			
10- tissues	"		**1,45**
fish 1			

[a)] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubation time:

- fish 1 viscera: 4h, fish 1 tissues: 23h
- fish 2 viscera: 23h45, fish 2 tissues: 45h30
- fish 3 viscera: 8 days 2h20, fish 3 tissues: 8 days 22h30
- fish 4 viscera: 17 days 23h, fish 4 tissues: 18 days 2h25.

[b)] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1h at 4°C, following a first extraction with acetone.

[c)] :Folch et al. 1957, following extractions with acetone, then ethanol..

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TABLE 7. EXTRACTION OF FRESH FISH LIPIDS (Trout)

Exp. No.	Technique	Yield (%)	Total (%)
1- viscera	acetone [a)]	34,70	
	ethanol [b)]	2,18	36,88
2- tissues	"	5,53	
		1,17	6,70
3- viscera	chlor:MeOH [c)]		39,81
4- tissues	"		14,93

Determinations in triplicates (variation < 5 %).
a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.
b) :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.
c) :Folch et al. 1957.

TABLE 8. EXTRACTION OF FRESH FISH LIPIDS (Herring)

Exp. No.	Technique	Yield (%)	Total (%)
1-tissues and viscera	acetone [a)]	2,09	
	ethanol [b)]	0,68	2,77
2-tissues and viscera	chlor:MeOH [c)]		5,95

Determination in triplicates (variation < 5 %).
a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°.
b) :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.
c) :Folch et al. 1957.

TABLE 9. EXTRACTION OF FRESH SHARK LIVER LIPIDS (M. schmitti)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a)]	36,39	
	ethyl acetate [b)]	4,48	40,87
2-	ethyl acetate [c)]		36,68
3-	chlor : MeOH [d)]		41,86

Determinations in triplicates (variations <5 %).
a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.
b) :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
c) :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
d) :Folch et al. 1957.

T⁴ ⁿLE 10. EXTRACTION OF FRESH SHARK LIVER LIPIDS (G. galeus).

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a)]	21,39	
	ethyl acetate [b)]	5,27	**26,66**
2-	ethyl acetate [c)]		**25,89**
3-	chlor : MeOH [d)]		**29,99**

Determinations in triplicates (variations <5 %).
a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.
b) :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
c) :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
d) :Folch et al. 1957.

TABLE 11. EXTRACTION OF FRESH SHARK LIVER LIPIDS (Angel Shark)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a)]	19,23	
	ethyl acetate [b)]	8,98	**28,21**
ʼ	ethyl acetate [c)]		**39,22**
3-	chlor : MeOH [d)]		**39,23**

Determinations in triplicates (variations <5 %).
a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.
b) :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
c) :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
d) :Folch et al. 1957.

TABLE 12. FATTY ACID COMPOSITION (E. pacifica)

Solvent	Saturated	Unsaturated Mono	Di	Poly	H-Poly	Unidentified
chlo-meth	26.18	22.54	1.91	3.23	26.34	19.8
acetone	21.4	22.18	1.75	3.7	24.52	26.46
acetone	19.09	22.11	2.03	3.48	30.24	23.03
ethanol	28.07	22.92	2.14	3.07	27.78	16.03
t-butanol	32.63	24.96	1.86	2.86	17.86	19.83
l acetate	22.68	25.77	2.17	2.88	22.98	23.51

..ta expressed in percentage of total fatty acids (%).

gy,

II b)

TABLE 13. CHARACTERISTICS OF KRILL OIL (*E. pacifica*)

		independent laboratory [a]	handbook[b]
Saponification index			
Fraction I [c]	130,6	—	—
Fraction II [d]	185,7	—	—
Olive oil	192,0 [e]	---	189,7
Wijs iodine index			
Fraction I [c]	185,2	172,5	—
Fraction II [d]	127,2	139,2	—
Olive oil	85,3 [e]	—	81,1
Cholesterol content (%)			
Fraction I [c]	2,1	1,9	—
Fraction II [d]	3,7	3,0	—
Olive oil	0,2 [e]	---	---
Volatile matter and moisture levels (%)			
Fraction I [c]	10,0	—	—
Fraction II [d]	6,8	---	—
Peroxide value (meq peroxide/kg oil)			
Fraction I [c]	—	0,0	—
Fraction II [d]	—	0,0	—
p-Anisidine value (g^{-1} absorption)			
Fraction I [c]	---	0,1	—
Fraction II [d]	---	5,5	---

[a]: Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
[b]: Harwood and Geyer 1964.
[c]: Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[d]: Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[e]: Extra virgin olive oil cold compressed from Bertolli ™.

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TABLE 14. LIPID CLASS COMPOSITION OF KRILL OIL (AREA %) (*E. pacifica*)

Triglycerides	
Fraction I [a]	19,0±0,7
Fraction II [b]	66,5± 2,3
Hydrocarbons	
Fraction I [a]	trace
Fraction II [b]	1,3± 0,1
Free fatty acids	
Fraction I [a]	23,7± 1,1
Fraction II [b]	20,3± 0,3
Monoglycerides	
Fraction I [a]	1,4± 0,3
Fraction II [b]	0,5± 0,1
Phospholipids or other polar material	
Fraction I [a]	54,1± 6,1
Fraction II [b]	8,5 ±1,6

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

[a] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 15. FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a]	Fraction II [b]
12 :0	0,0	0,1
13 :0	0,2	0,1
ISO 14 :0	0,4	0,6
14:0	4,2	7,6
ISO 15:0	0,5	0,7
ANT 15:0	0,2	0,2
15:0	0,6	1,0
ISO 16:0	0,2	0,3
ANT 16:0	0,2	0,2
16:0	14,1	21,6
7MH	0,6	0,9
ANT 17:0	0,1	0,3
17:0	2,8	3,7
18:0	1,0	1,6
20:0	0,1	0,3
Saturates	25,2	39,2

TABLE 15 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%)
(*E. pacifica*)

Fatty acids	Fraction I [a]	Fraction II [b]
14:1	0,4	0,5
15:1	0,1	0,2
16:1 n-7	6,6	7,8
16:1 n-5	0,6	0,2
17:1	0,6	0,7
18:1 n-9	8,0	9,8
18:1 n-7	4,2	5,6
18:1 n-5	0,1	0,1
20:1 n-9	0,3	0,4
20:1 n-7	0,3	0,4
20:1 n-5	0,3	0,4
22:1 n-11 +13	0,1	0,2
Monoenes	21,6	26,3
16:2 n-6	0,6	1,2
16:2 n-4	1,3	1,3
18:2 n-7	0,1	0,2
18:2 n-6	2,0	1,8
18:2 n-4	0,1	0,1
20:2 NMID	0,2	0,2
20:2 n-6	0,1	0,1
Dienes	4,4	4,9
16:3 n-4	1,4	1,2
18:3 n-6	0,4	0,3
18:3 n-4	0,2	0,2
18:3 n-3	3,2	3,0
18:3 n-1	0,1	0,1
20:3 n-3	0,1	0,1
Trienes	5,4	4,9
16:4 n-3	0,9	0,7
16:4 n-1	1,0	0,8
18:4 n-3	9,2	7,4
18:4 n-1	0,1	0,0
20:4 n-6	0,7	0,5
20:4 n-3	0,7	0,3
Tetraenes	12,6	9,7
20:5 n-3	17,4	8,6
21:5 n-3	0,7	0,5
22:5 n-6	0,2	0,1
22:5 n-3	0,5	0,3
Pentaenes	18,8	9,5

TABLE 15 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
22:6 n-3	13,2	6,6
Hexaenes		
Iodine value calculated	214,8	145,1

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 16. KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
12:0	0,5	0,1
13:0	0,2	0,0
ISO14:0	0,2	0,2
14:0	1,3	2,6
ISO 15:0	0,3	0,3
ANT 15:0	0,1	0,1
15:0	0,2	0,5
ISO 16:0	0,1	0,2
ANT 16:0	0,2	0,1
16:0	3,3	10,6
7MH	0,6	0,8
ANT 17:0	0,2	0,2
Phytanic	0,2	0,0
17:0	0,5	0,8
18:0	0,2	0,6
20:0	0,3	0,2
22:0	0,0	0,1
Saturates	8,4	17,4
14:1	0,2	0,2
15:1	0,2	0,1
16:1 n-9	0,5	0,0
16:1 n-7	5,2	6,8
16:1 n-5+I17:0	0,1	0,1
17:1	0,6	0,7
18:1 n-9	7,0	11,4
18:1 n-7	4,9	9,3
18:1 n-5	0,1	0,3
20:1 n-11	0,2	0,3
20:1 n-9	0,1	0,3
22:1 n-11+13	0,1	0,2
24:1 n-9	0,0	0,1
Monoenes	19.2	29.8

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TABLE 16 (continued). KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
16:2 n-6	0,4	0,9
16:2 n-4	1,2	1,0
18:2 n-7	0,1	0,2
18:2 n-6	2,4	2,6
18:2 n-4	0,1	0,1
20:2 n-6	0,1	0,1
Dienes	4,3	4,9
16:3 n-4+I17:1	1,4	0,9
16:3 n-3+I18:0	0,2	0,5
18:3 n-6	0,4	0,3
18:3 n-4	0,1	0,1
18:3 n-3	3,3	3,4
18:3 n-1	0,1	0,1
20:3 n-6	0,1	0,1
20:3 n-3	0,1	0,2
Trienes	5,7	5,6
16:4 n-3	0,6	0,3
16:4 n-1	1,0	0,6
18:4 n-3	9,8	6,2
18:4 n-1	0,1	0,1
20:4n-6	1,7	1,4
20:4 n-3	0,6	0,5
22:4 n-3	0,3	0,3
Tetraenes	14,1	9,4
18:5 n-3	0,2	0,1
20:5 n-3	26,4	17,4
21:5 n-3	0,9	0,6
22:5 n-6	0,0	0,1
22:5 n-3	0,7	0,5
Pentaenes	28,2	18,7
22:6 n-3	20,5	14,4
Hexaenes	20,5	14,4
Iodine value calculated	291,6	220,3

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

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TABLE 17. TOCOPHEROL, ALL-*trans* RETINOL AND CHOLECALCIFEROL CONTENT IN KRILL OIL (*E. pacifica*)

alpha-tocopherol by HPLC (IU)	
Fraction I [a]	0,91
Fraction II [b]	0,83
gamma-tocopherol by HPLC µg/g	
Fraction I [a]	Tr
Fraction II [b]	Tr
delta-tocopherol by HPLC µg/g	
Fraction I [a]	N.D.
Fraction II [b]	N.D.
all-*trans* retinol by HPLC (IU)	
Fraction I [a]	395,57
Fraction II [b]	440,47
cholecalciferol by HPLC (IU)	
Fraction I [a]	N.D.
Fraction II [b]	N.D.

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
Data expressed per gram of krill oil.
[a] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[b] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
TR = trace
N.D. = not detected

Conversion : Vitamin	alpha-tocopherol	mg/g oil x 1,36 = International Unit
	All-*trans* retinol	µg/g ÷ 0,3 = International Unit

TABLE 18. ASTAXANTHIN AND CANTHAXANTHIN CONTENT OF KRILL OIL (*E. pacifica*)

Asthaxantin (µg/g oil)	
Fraction I [a]	93,1
Fraction II [b]	121,7
Canthaxanthin (µg/g oil)	
Fraction I [a]	270,4
Fraction II [b]	733,0

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
[a] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[b] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

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TABLE 19. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggested procedure)

STEP	CONDITIONS
Grinding (if particles > 5mm)	4°C
Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min) 4°C
Filtration	organic solvent resistant filter under reduced pressure
Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure
Oil-water separation	4°C
Lipid extraction	sample: ethyl acetate ratio of 1:2 (w/v)[a)] pure ethyl acetate 30 min 4°C [b)]
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

[a)]: Ethanol can be replaced by isopropanol, *t*-butanol or ethyl acetate.
[b)]: 25 °C when using *t*-butanol.

TABLE 20: PROTEOLYTIC ACTIVITY OF KRILL RESIDU USING LACTOSERUM AS THE SUBSTRATE, AT 37 °C, PH 7.0 FOR A RATIO ENZYME:SUBSTRATE OF 1:43

Time (min)	Amino acids released (μmoles)	Enzymatic rate (μmoles/min)	Specific enzymatic activity (μmoles/min/mg*)
15	28.76	1.917	0.164
30	43.74	0.999	0.125
170	98.51	0.322	0.050
255	177.26	0.308	0.060

* total quantity of enzymes in hydrolysis media

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We claim:

1. A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:
   (a) placing marine and aquatic animal material in a ketone solvent, preferably acetone to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;
   (b) separating the liquid and solid contents;
   (c) recovering a first lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;
   (d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol, preferably ethanol, isopropanol or *t*-butanol and esters of acetic acid, preferably ethyl acetate to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;
   (e) separating the liquid and solid contents;
   (f) recovering a second lipid rich fraction by evaporation of the solvent from the liquid contents;
   (g) recovering the solid contents.

2. A method as in claim 1 wherein during step (a), the solvent and animal material are homogenized.

3. A method as in claim 1 wherein during step (d), the solvent and solid contents are homogenized.

4. A method as in any of claims 1 to 3 wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. A method as in any of claims 1 to 4 wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. A method as in any of claims 1 to 5 wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

5 7. A method as in any of claims 1 to 6 wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

10 8. A method as in any of claims 1 to 7 wherein after step (e) and before step (f), the method additionally comprises the intervening step of washing the solid contents with the organic solvent selected in step (d).

9. A method as in any of claims 1 to 8 wherein prior to step (a) the marine and
15 aquatic animal material is finely divided, preferably to an average particle size of 5mm or less.

10. A method as in claims 1 to 9 wherein steps (a) and (b) are conducted at solvent temperatures of about 5°C or less.

20

11. A method as in claims 1 to 10 wherein said marine and aquatic animal is zooplancton.

12. A method as in claim 11 wherein said zooplancton is krill.

25

13. A method as in claim 12 wherein said zooplancton is *Calanus*.

14. A method as in claims 1 to 10 wherein said marine and aquatic animal is fish filleting by-products.

30

15. A method for extracting lipid fractions from marine and aquatic animal material selected from zooplancton and fish filleting by-products, preferably viscera, said method comprising the steps of:
    (a) placing said animal material in a ketone solvent, preferably acetone to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;
    (b) separating the liquid and solid contents;
    (c) recovering a lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;
    (d) recovering the solid contents.

16. A method as in claim 15 wherein the animal material is krill.

17. A method as in claim 15 wherein the animal material is *Calanus*.

18. A method as in claims15 to 17 wherein during step (a), the solvent and animal material are homogenized.

19. A method as in any of claims 15 to 18 wherein steps (b) and (d) are conducted under inert gas atmosphere.

20. A method as in any of claims 15 to 19 wherein step (b) is effected by techniques selected from filtration, centrifugation and sedimentation.

21. A method as in any of claims 15 to 20 wherein step (c) is effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

22. A method as in any of claims 15 to 21 wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

23. A method as in any of claims 15 to 22 wherein prior to step (a) the marine and aquatic animal material is finely divided, preferably to an average particle size of 5mm or less.

5

24. A method as in claims 15 to 23 wherein steps (a) and (b) are conducted at solvent temperatures of about 5°C or less.

25. A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 and preferably at least about 90:g/g of krill extract.

10

26. A krill lipid extract characterized in that the carotenoid content in canthaxanthin is as least about 250 µg/g and preferably at least about 270 µg/g of krill extract.

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27. A method of lipid extraction as in claims 1 or 15 wherein the solid contents recovered in the last step consists of a dehydrated residue containing active enzymes.

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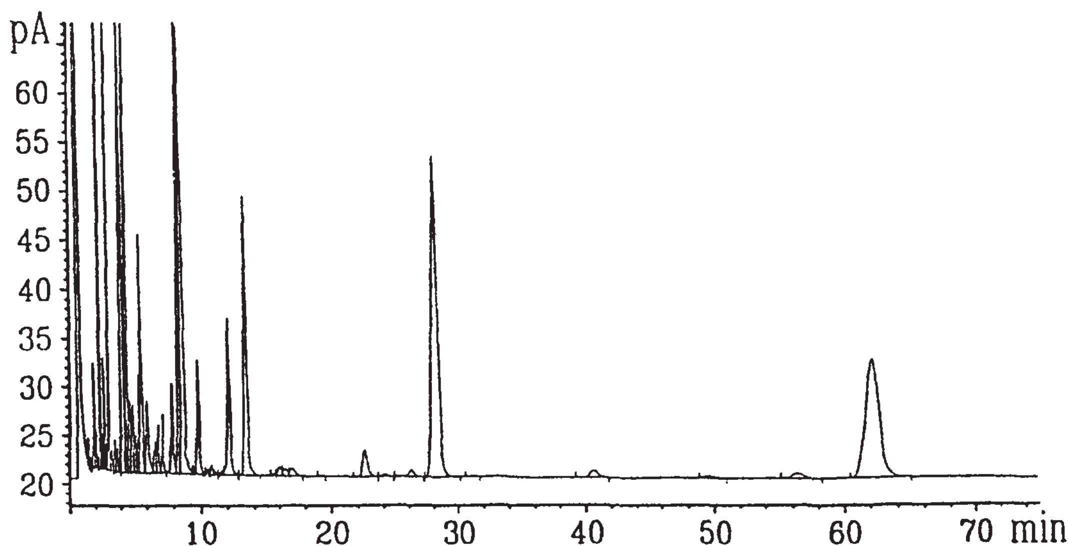

FIG. 1

1.263	4.521 - 16:1	11.637
1.455 - 12:0	4.684	12.145 - 18:3
1.625	4.891 - 16:1tr	13.458
1.812	5.121	15.626 - 20:0
1.876	5.426	16.045
2.056	5.570	16.482 - 20:1
2.173	6.037	17.017 - 20:1(cis11)
2.331 - 14:0	6.662	19.344 - 20:2
2.505	6.871	22.606 - 20:4(6,10,14,18)
2.591 - 14:1	7.235	24.103
2.682	7.925 - 18:0	26.247
2.802	8.439 - 18:1	28.287
2.855	8.640 - 18:1tr	31.295
3.078 - std 15:0	9.544	40.655
3.309	9.801 - 18:2	49.721
3.586	10.491	56.373
3.810	10.825	62.225
4.176 - 16:0	11.042	

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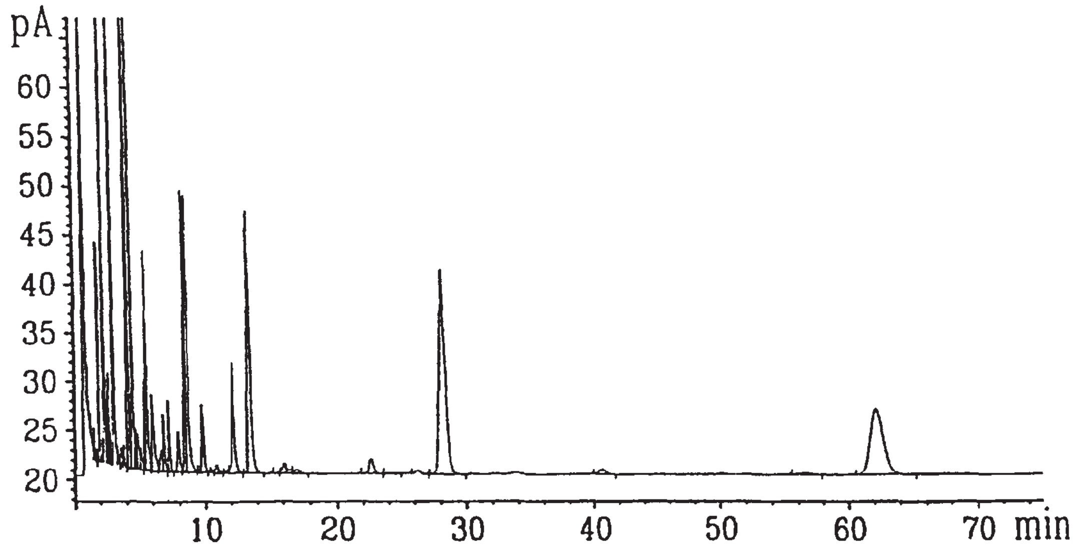

FIG. 2

1.217	3.806	9.786 - 18:2
1.264	4.157 - 16:0	10.484
1.454 - 12:0	4.515 - 16:1	10.813
1.624	4.680	11.590
1.812	4.891 - 16:1tr	12.136 - 18:3
1.876	5.028	13.447
2.055	5.109	15.623 - 20:0
2.171	5.421	16.025
2.330 - 14:0	5.562	16.466 - 20:1
2.505	6.031	17.021 - 20:1 (cis11)
2.591 - 14:1	6.642	22.585 - 20:4 (6,10,14,18)
2.680	6.870	24.100
2.800	7.230	26.217
2.854	7.910 - 18:0	28.241
3.077 - std 15:0	8.419 - 18:1	40.622
3.306	8.622 - 18:1tr	56.417
3.585	9.529	62.086

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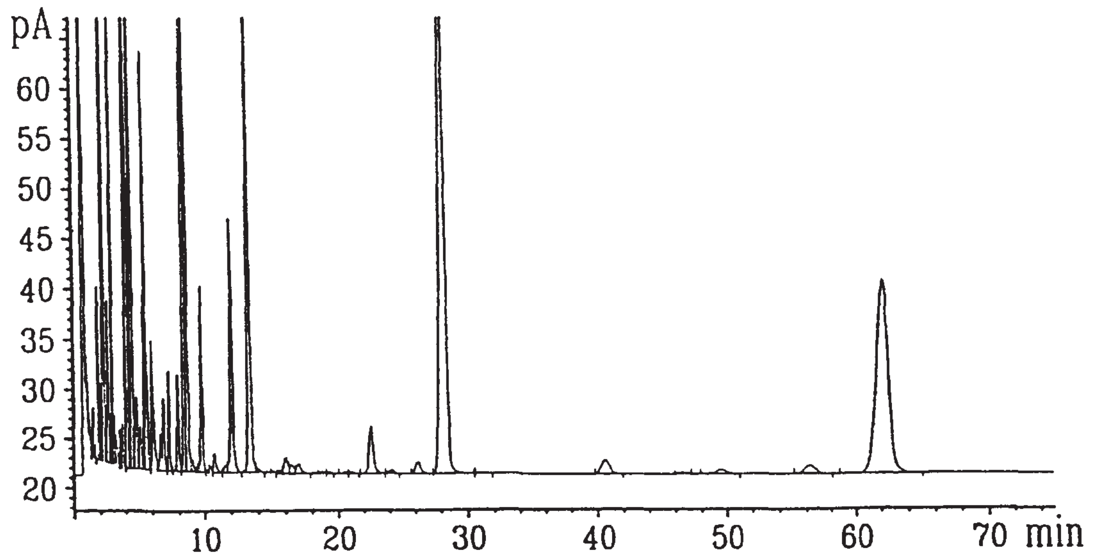

FIG. 3

1.216
1.262
1.454 - 12:0
1.624
1.811
1.875
2.016
2.054
2.174
2.330 - 14:0
2.505
2.589 - 14:1
2.679
2.799
2.854
2.981
3.074 - std 15:0
3.304
3.580
3.804
4.169 - 16:0
4.296

4.520 - 16:1
4.683
4.884 - 16:1tr
5.030
5.111
5.420
5.561
6.031
6.642
6.868
7.226
7.908 - 18:0
8.444 - 18:1
8.639 - 18:1tr
9.005
9.536
9.788 - 18:2
10.267
10.481
10.807
11.626
12.140 - 18:3

13.457
13.943
15.053
15.572 - 20:0
16.016
16.486 - 20:1
16.999 - 20:1(cis11)
18.762
19.303 - 20:2
20.474
21.027 - 20:3
22.575 - 20:4 (6,10,14,18)
24.071
26.215
28.333
31.180
40.560
46.595
49.513
56.292
62.250

4/20

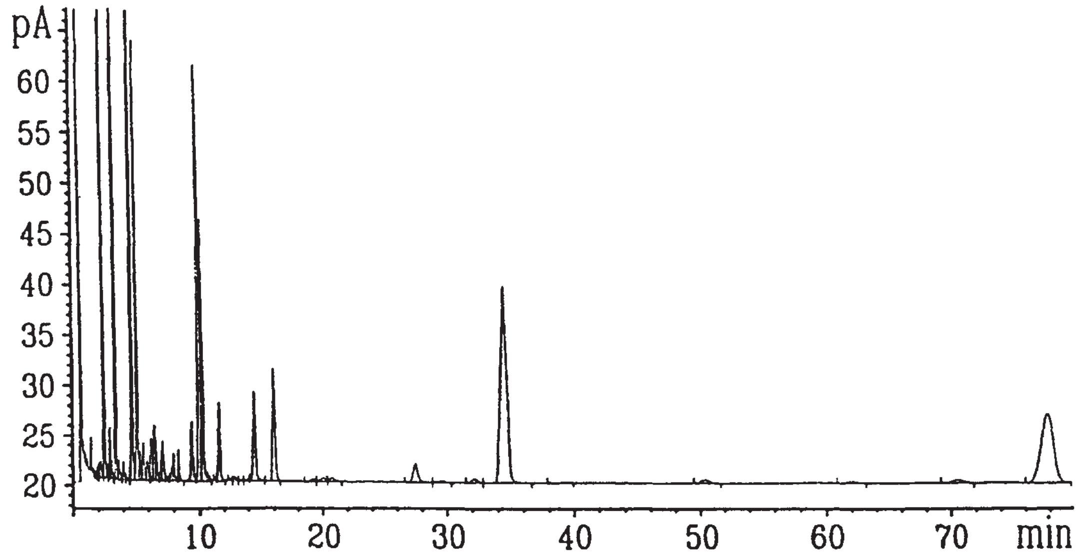

FIG. 4

1.552 - 12:0	5.675 - 16:1tr	12.888
1.749	5.964	13.388
1.968	6.284	14.017
2.095	6.533	14.524 - 18:3
2.262	6.655	16.107
2.485	7.009	19.275 - 20:0
2.582 - 14:0	7.159	20.112 - 20:1 (cis11)
2.784	7.440	20.781 - 20:1
2.886 - 14:1	7.874	27.553
3.004	8.019	29.529
3.145	8.462	32.161
3.478 - std 15:0	9.411 - 18:0	34.614
3.720	10.000 - 18:1	39.240
4.088	10.249 - 18:1tr	50.374
4.325	10.716	61.892
4.793 - 16:0	11.357	70.568
5.196 - 16:1	11.647 - 18:2	77.894
5.406	12.519	

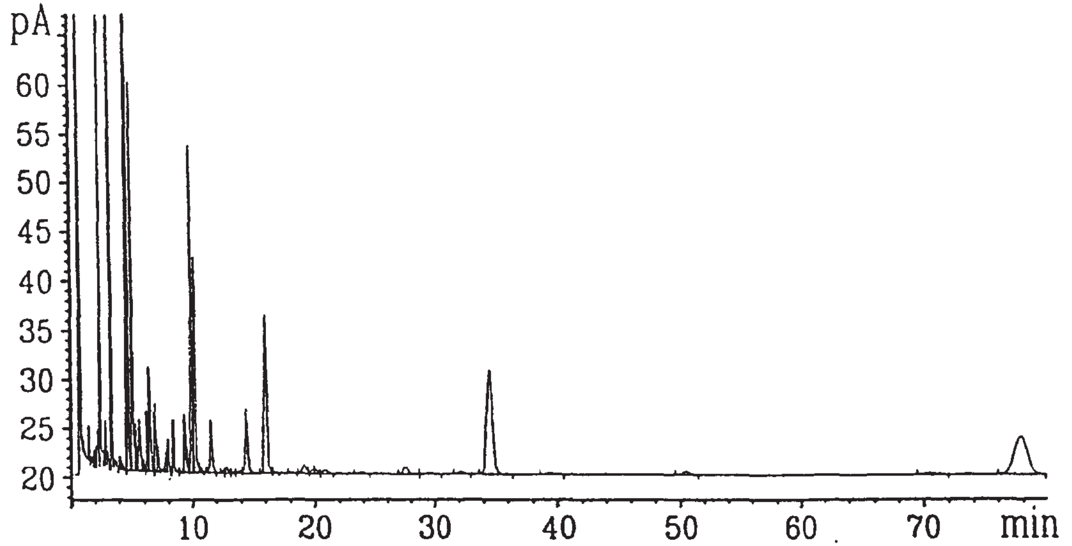

FIG. 5

1.556 – 12:0	5.977	14.540 – 18:3
1.753	6.294	16.133
1.972	6.546	16.805
2.104	7.020	18.131
2.304	7.166	19.154 – 20:0
2.590 – 14:0	7.889	19.875
2.892 – 14:1	8.030	20.099 – 20:1(cis11)
3.012	8.473	20.820 – 20:1
3.153	9.425 – 18:0	23.903
3.485 – std 15:0	10.010 – 18:1	27.583
3.710	10.260 – 18:1tr	29.570
4.096	10.735	32.195
4.203	11.394	34.597
4.333	11.661 – 18:2	39.334
4.800 – 16:0	12.540	50.452
5.206 – 16:1	12.909	70.660
5.417	13.402	77.895 – 24:0
5.711 – 16:1tr	14.010	

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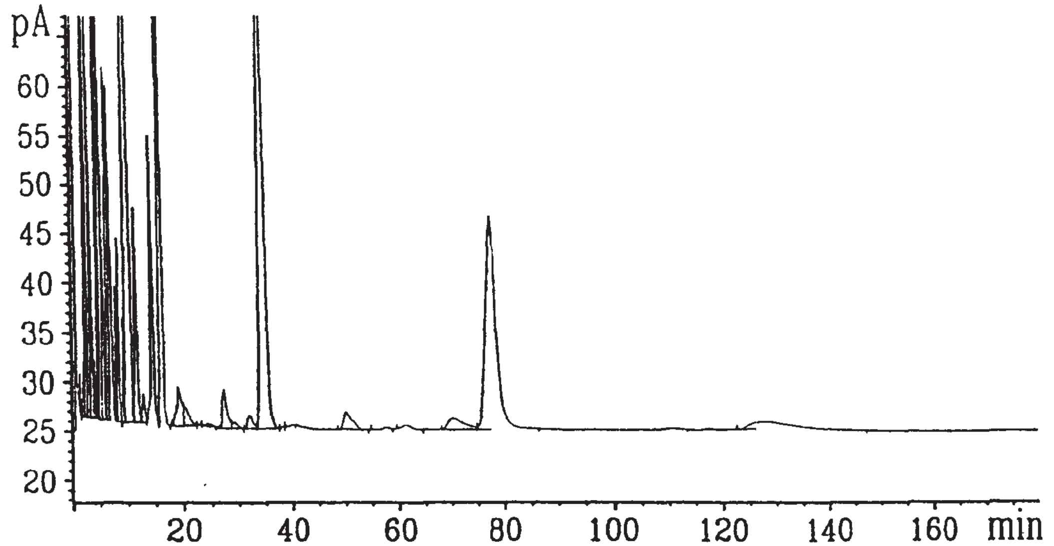

FIG. 6

1.564 – 12:0	8.025	34.677
1.762	8.485	38.373 – 22:0
1.977	9.360 – 18:0	40.163
2.080	9.407	50.157
2.273	10.147 – 18:1tr	57.532
2.591 – 14:0	11.618 – 18:2	61.436
2.887 – 14:1	12.858	70.271
3.008	14.515 – 18:3	77.784 – 24:0
3.470 – std 15:0	16:162	110.694
4.108	18.077	127.696
4.341	19.355 – 20:1	
4.803 – 16:0	20.182 – 20:4 (cis11)	
5.210 – 16:1tr	20.311	
5.683	23.205 – 20:2	
6.292	24.678	
6.514	27.411 – 20:4 (6,10,14,18)	
7.030	29.307	
7.810	31.990	

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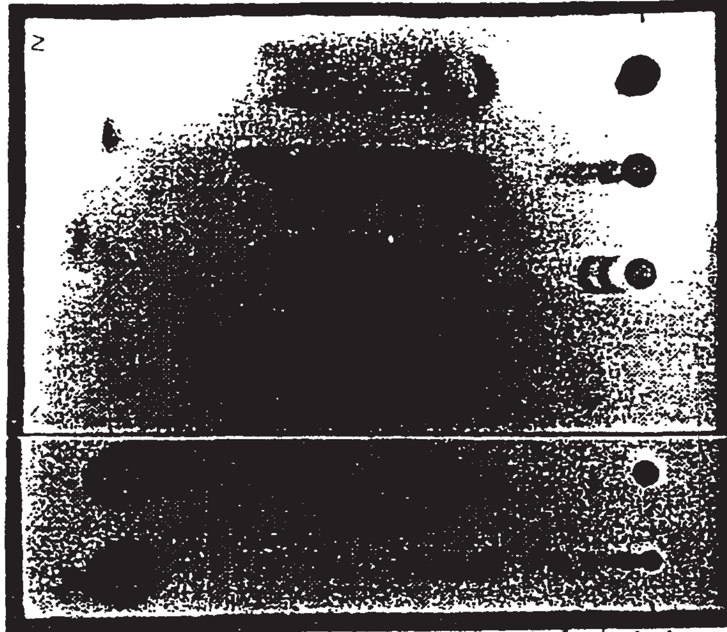

FIG. 7

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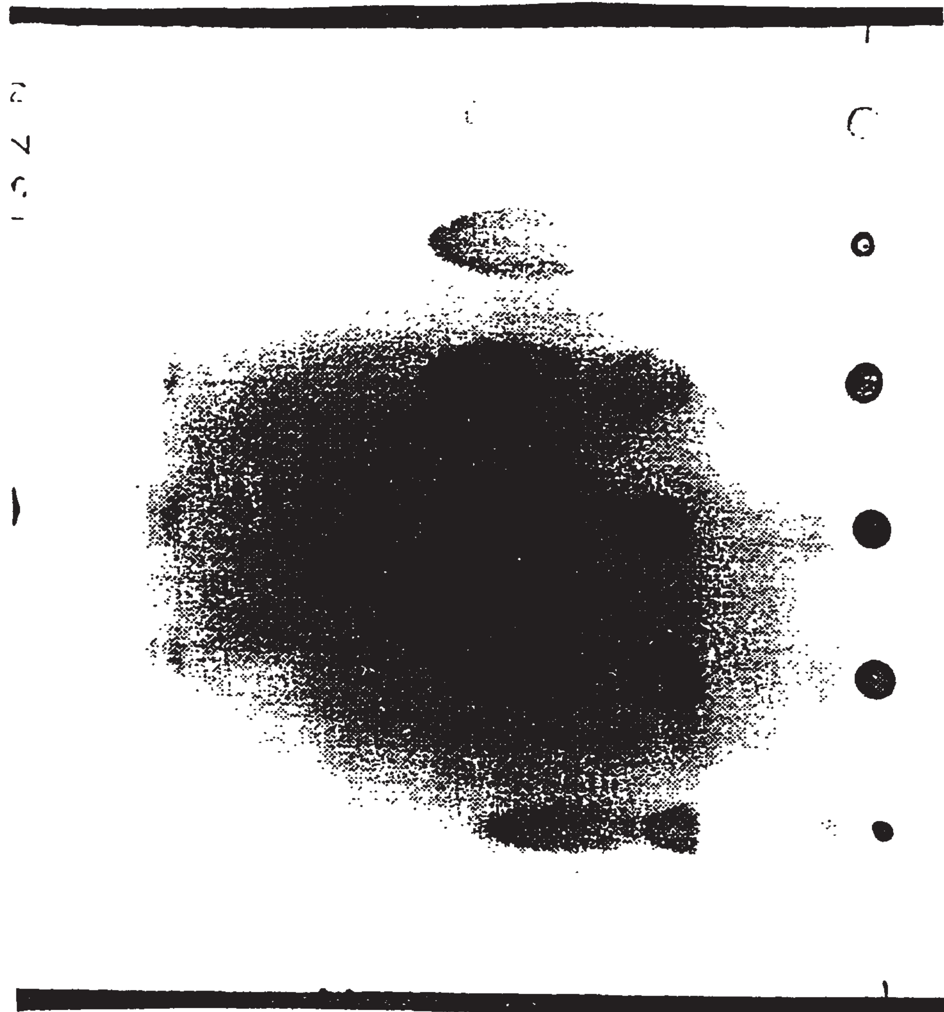

FIG. 8

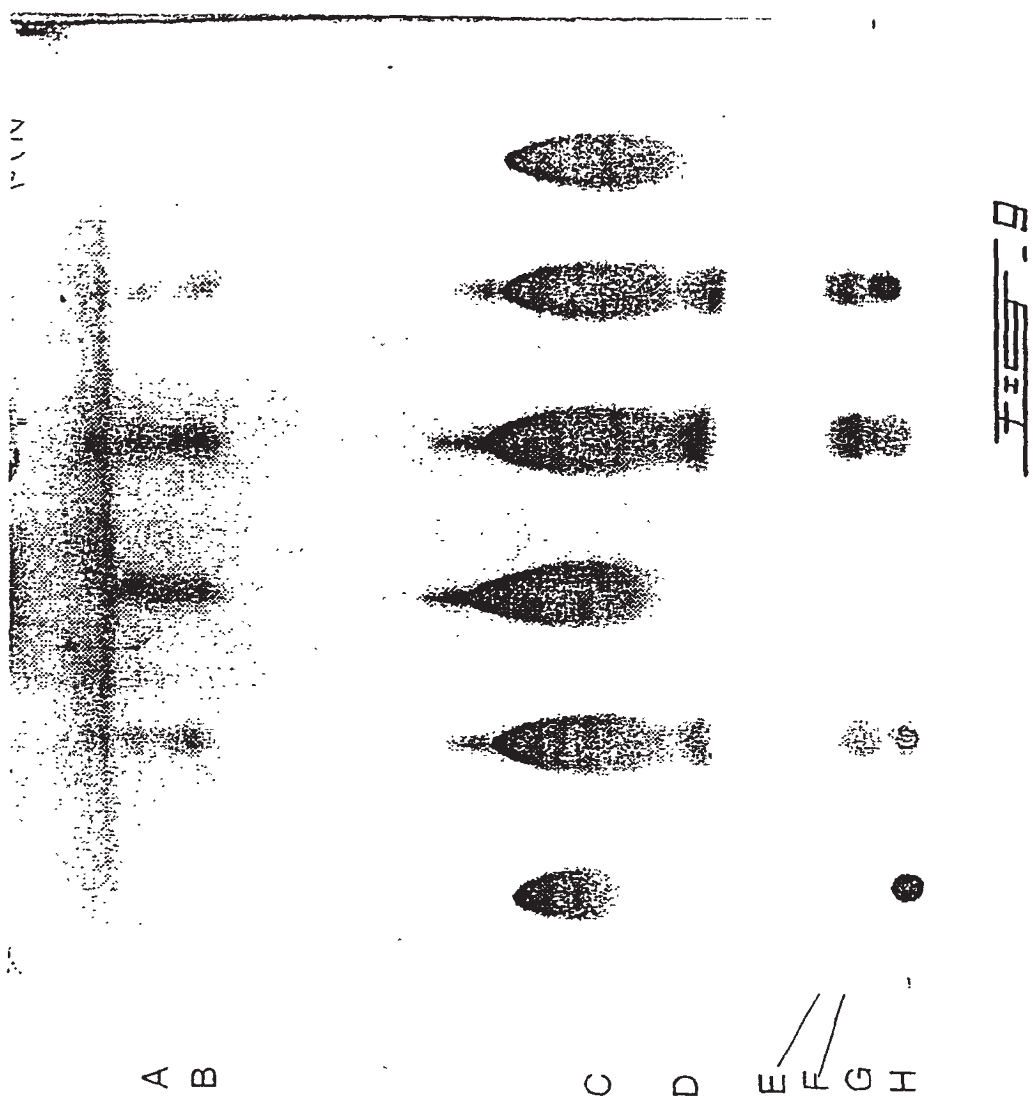

FIG. 9

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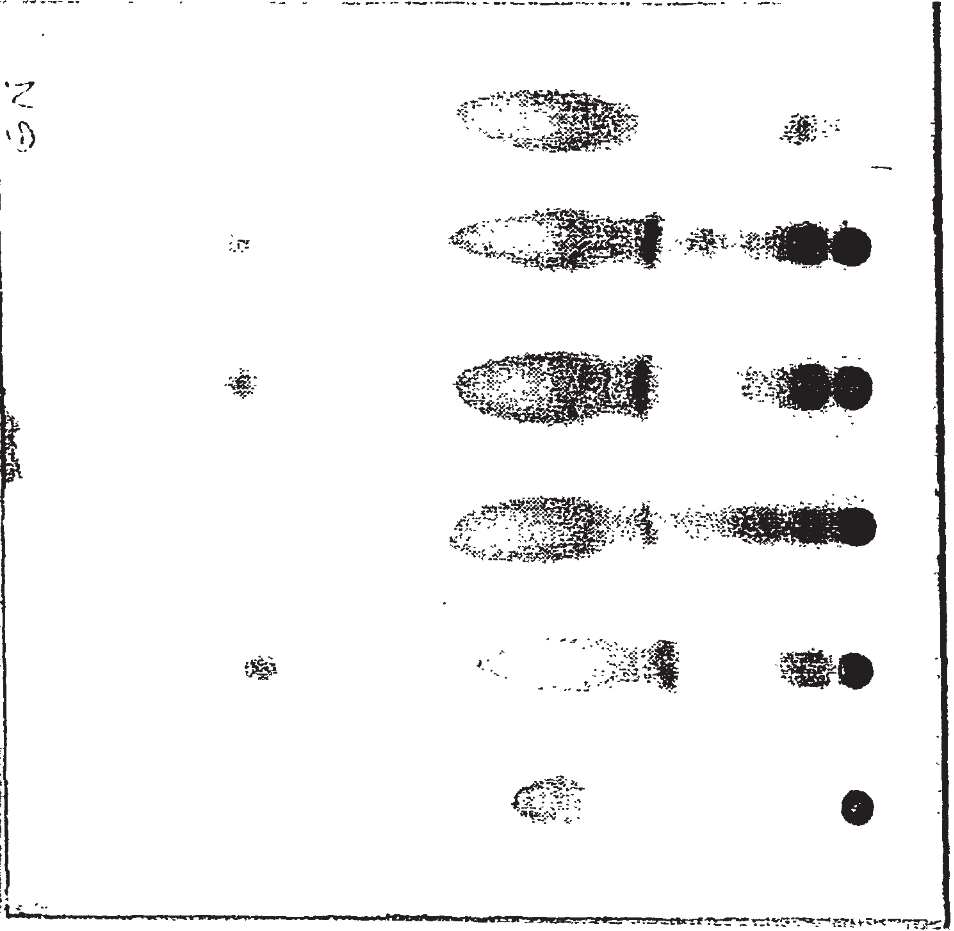

SUBSTITUTE SHEET (RULE 26)

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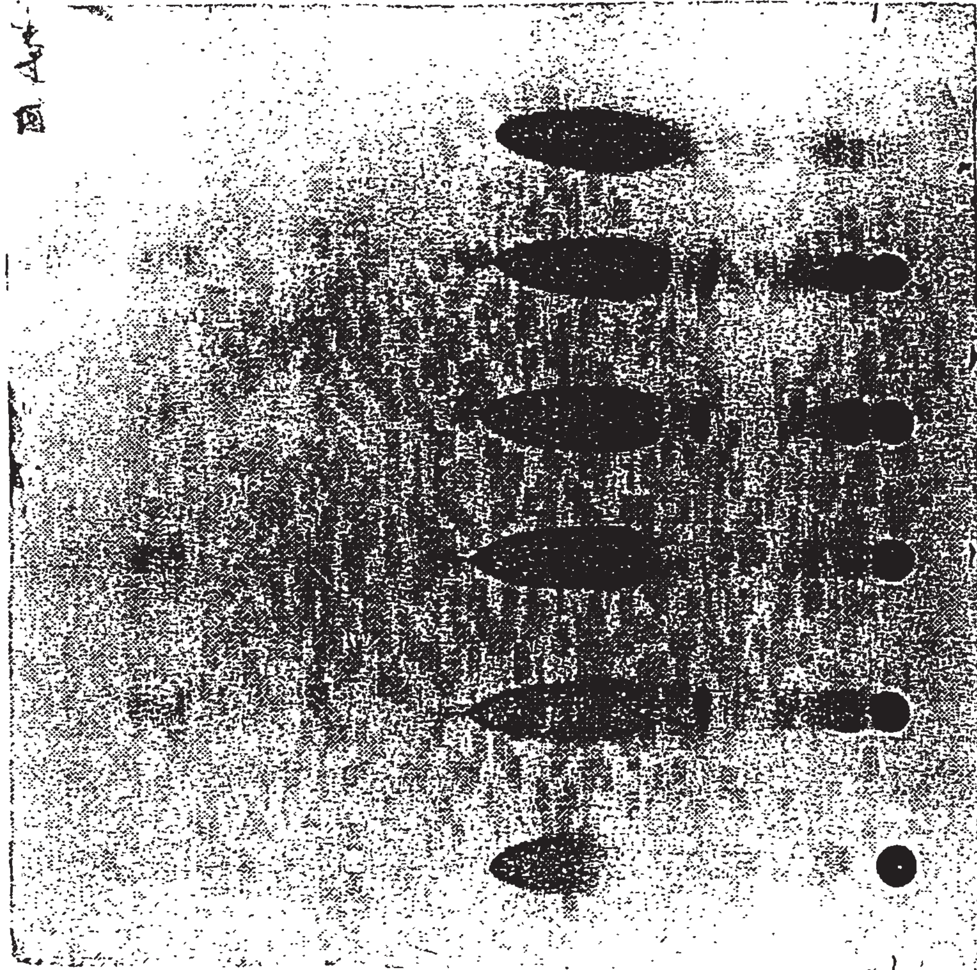

Fig. 11

A B C D E F G H

SUBSTITUTE SHEET (RULE 26)

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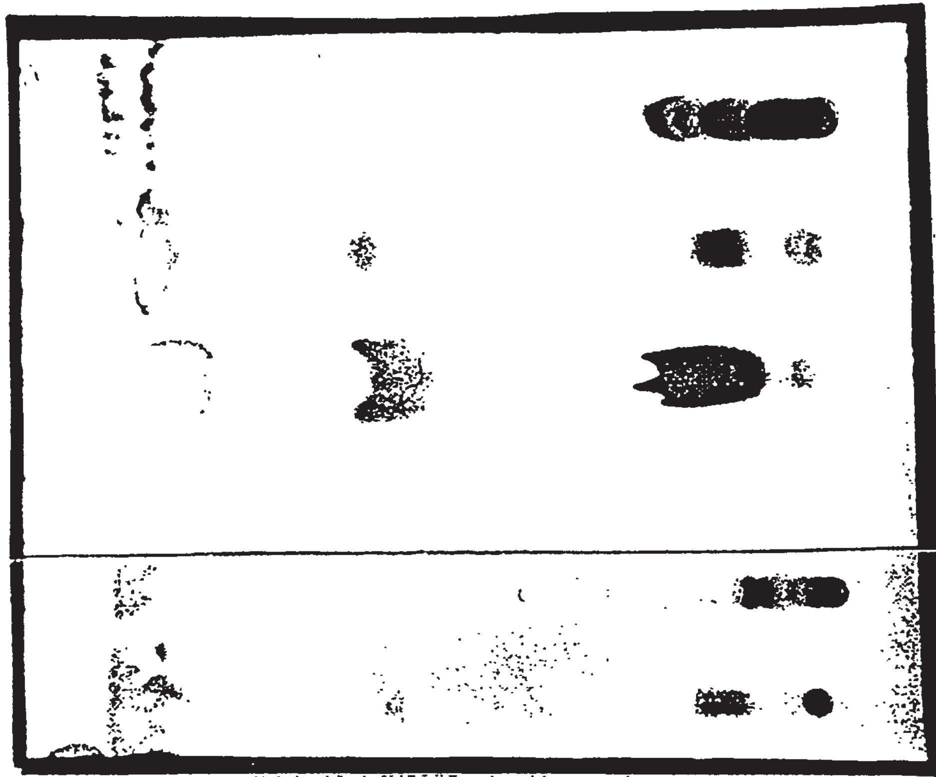

Neutral lipids

Cephalin

Lecithin
Sphingomyelin
Lysolecithin
Origin

FIG. 12

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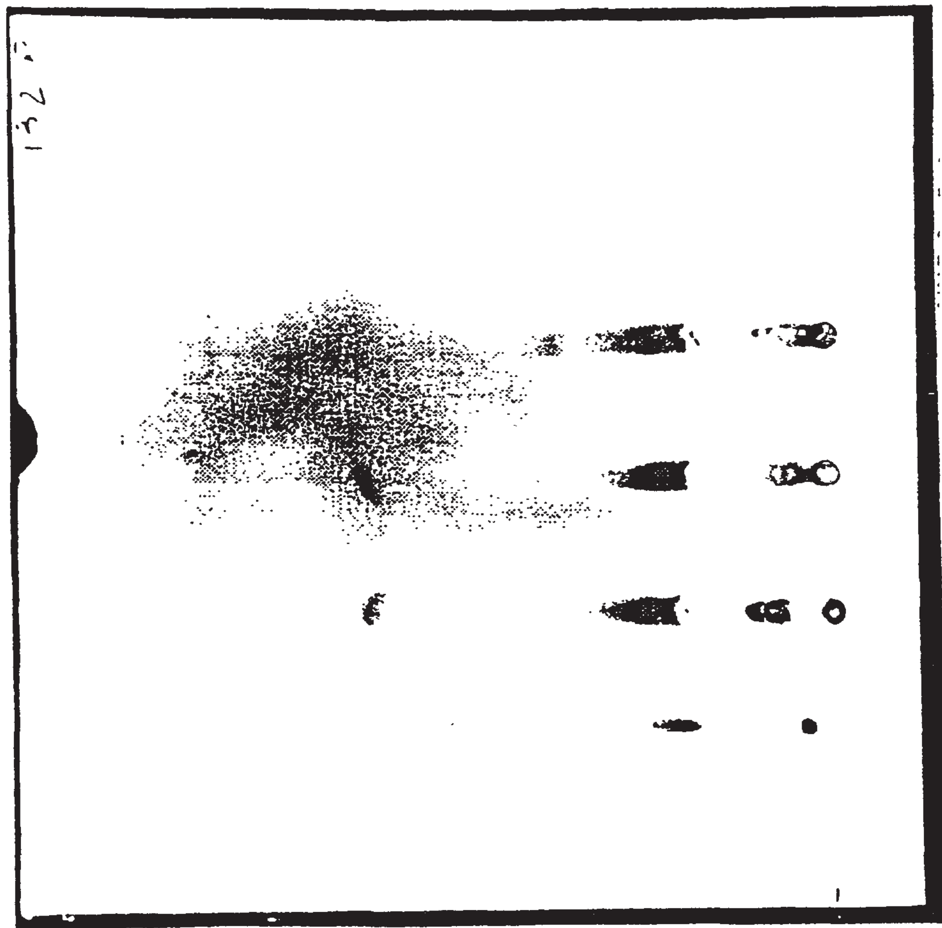

Neutral lipids

Cephalin

Lecithin

Sphingomyelin

Lysolecithin

Origin

FIG. 13

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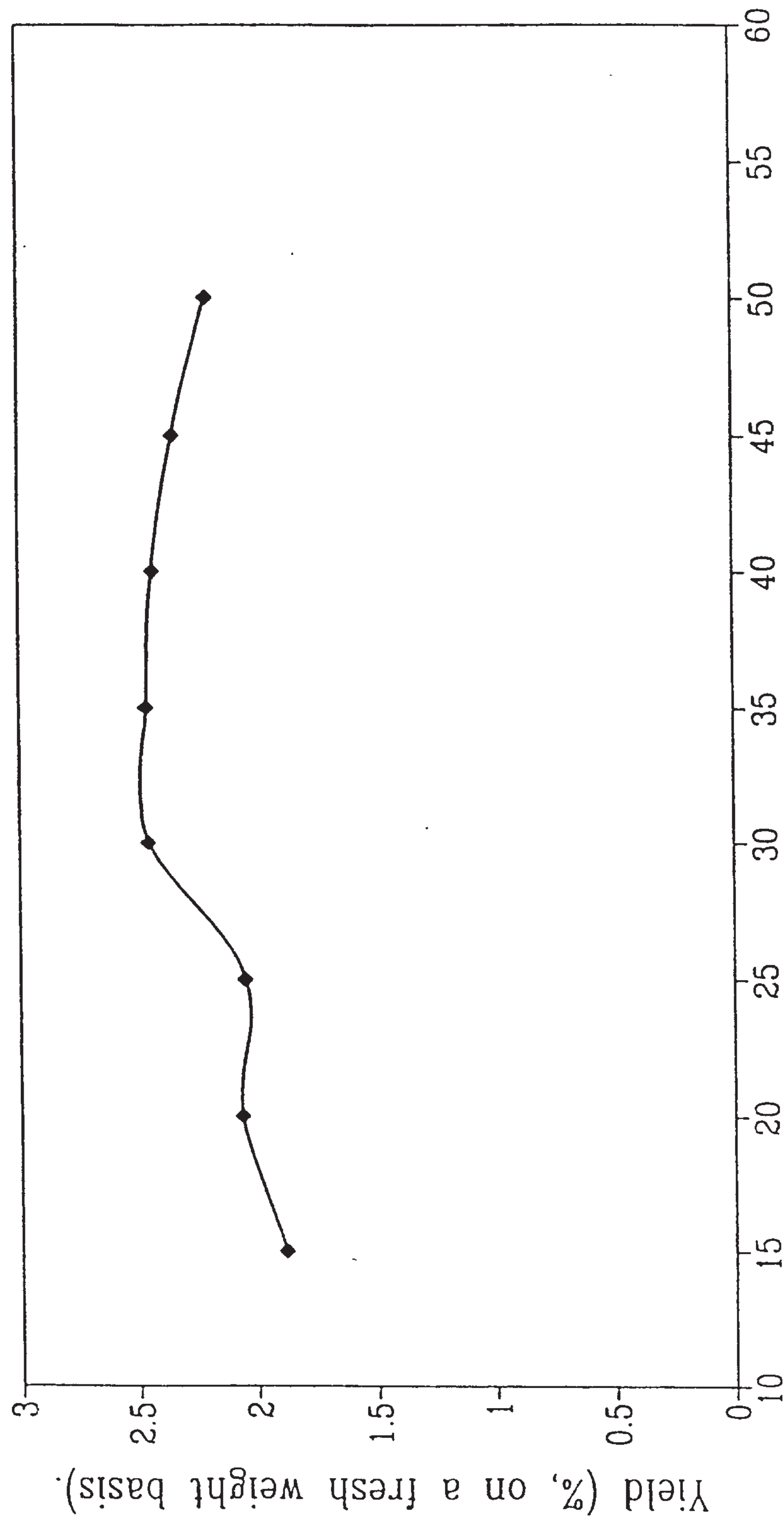
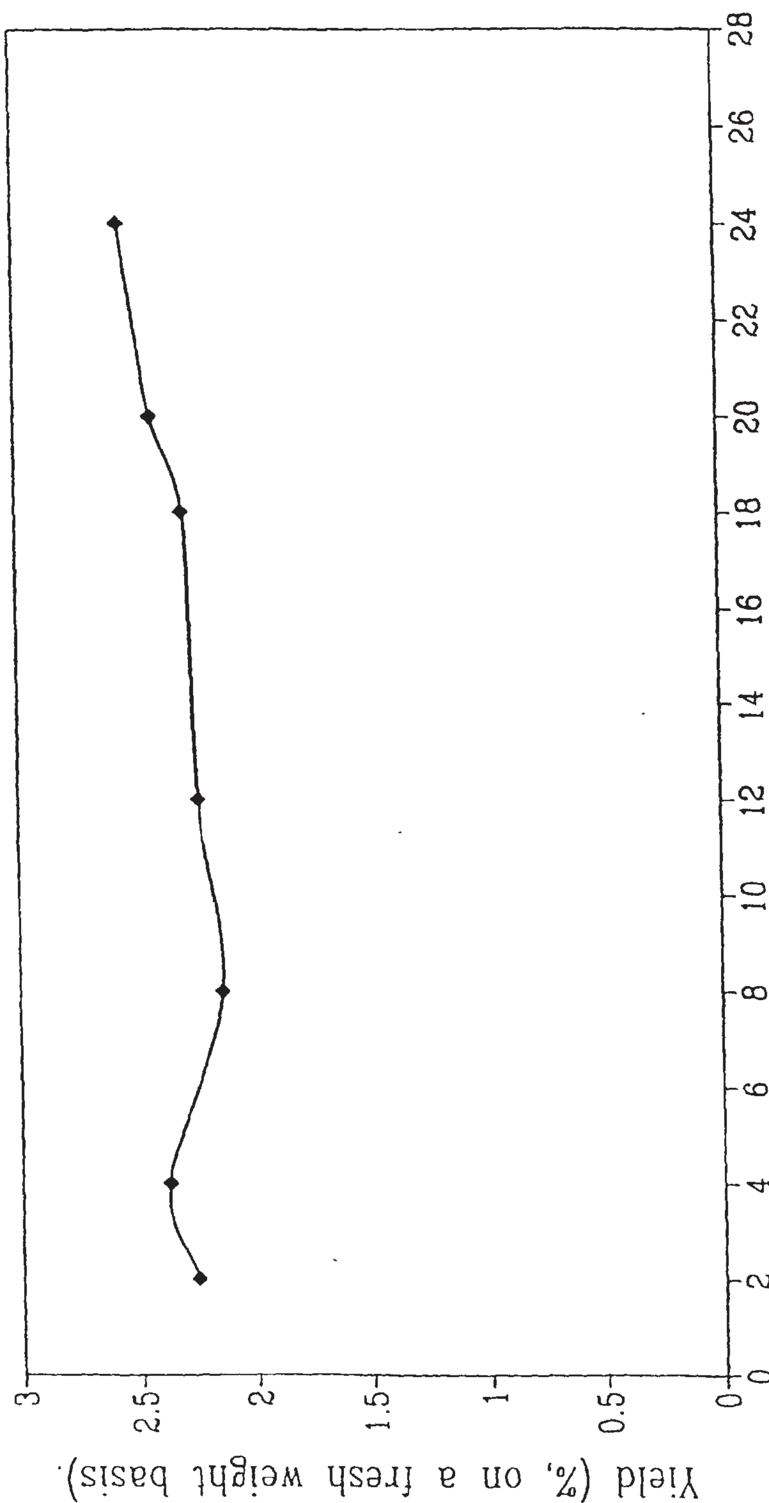


Yield (%, on a fresh weight basis).

Volume of acetone (mL).
Incubation time of 2 h.
Determinations in triplicates (variation less than 5 %).

FIG. 17



Incubation time in acetone (h).
Sample-acetone ratio of 1:9 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 1B

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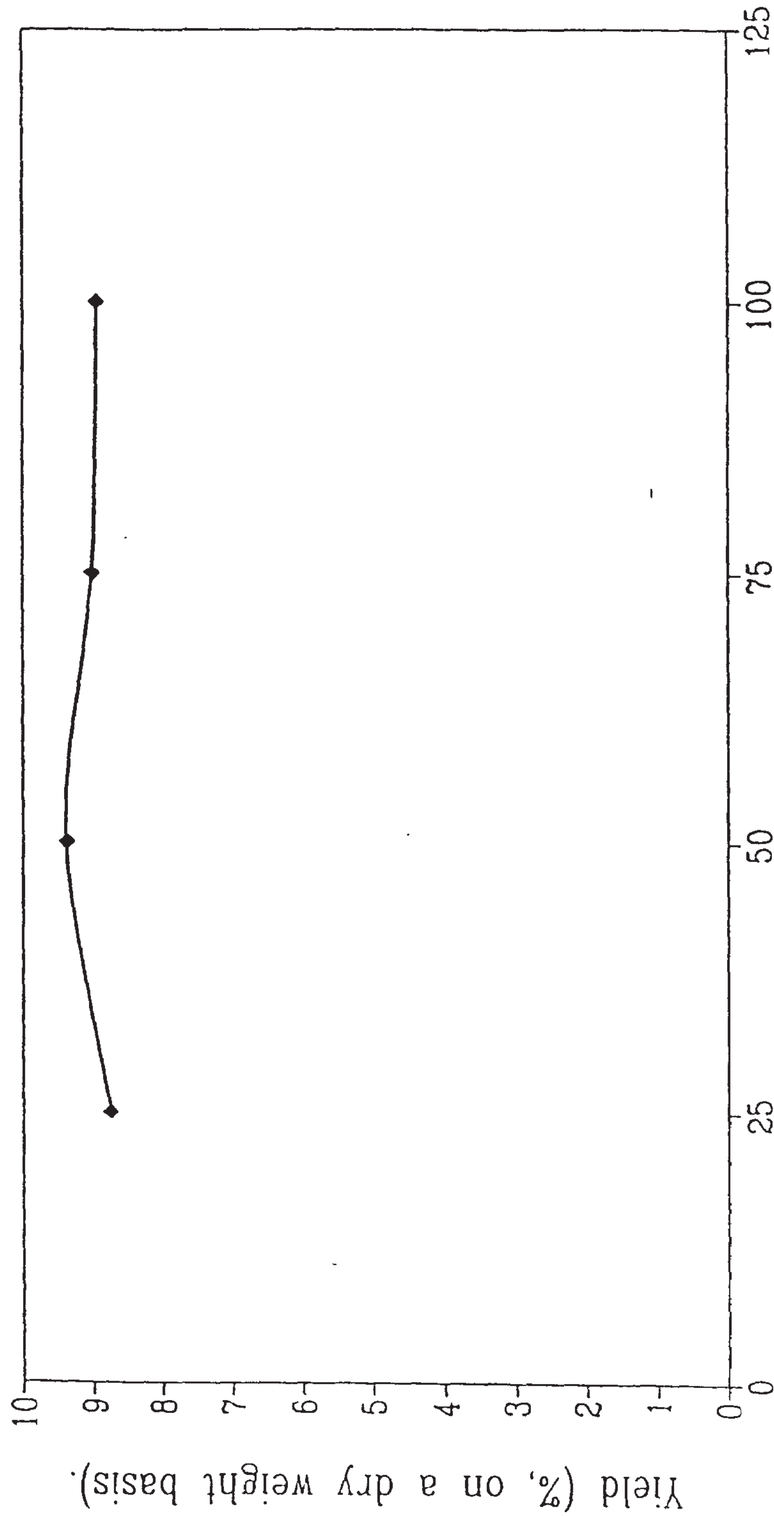

Volume of ethanol (mL).
Incubation time of 30 min.
Determinations in triplicates (variation less than 5 %).

FIG-19

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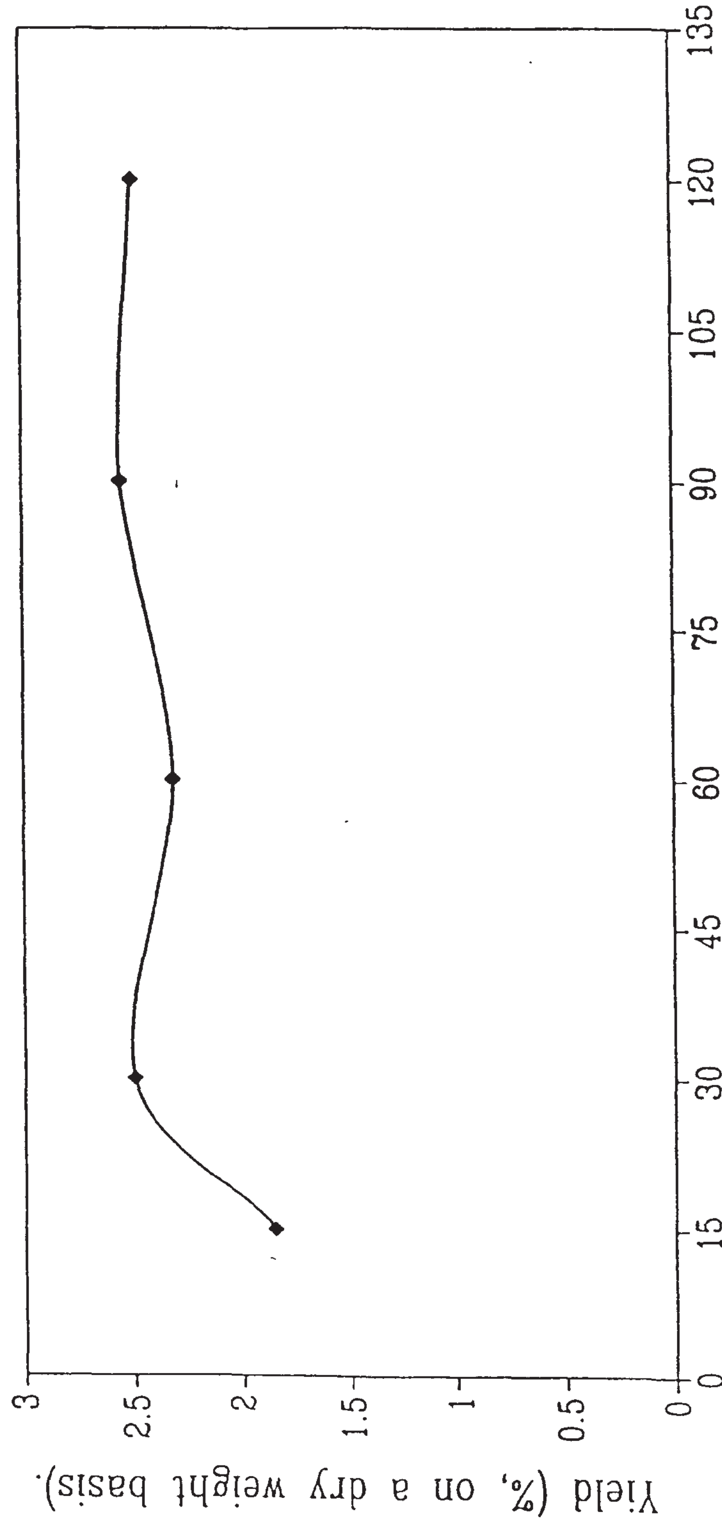

Incubation time in ethanol (min).
Sample-ethanol ratio of 1:4 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/00987

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C11B1/10 C12N9/64

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)
IPC 7 C11B C12N

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 12, 26 December 1996 (1996-12-26) -& JP 08 198754 A (YAKULT HONSHA CO LTD), 6 August 1996 (1996-08-06) abstract	1,2,5, 14,15, 18,20
Y		27
Y	WO 84 01715 A (HELLGREN LARS G I ;MOHR VIGGO (NO); VINCENT JAN GUSTAV (SE)) 10 May 1984 (1984-05-10) page 7, line 29 - line 31 examples 1,2	27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2000	28/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Dekeirel, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 197845 Derwent Publications Ltd., London, GB; Class C03, AN 1978-80681A XP002129262 -& JP 53 112195 A (NIPPON PAINT CO LTD), 30 September 1978 (1978-09-30) abstract	15
X	PATENT ABSTRACTS OF JAPAN vol. 009, no. 160 (C-289), 4 July 1985 (1985-07-04) -& JP 60 035057 A (SANEI KAGAKU KOGYO KK), 22 February 1985 (1985-02-22) abstract	15,16,25
X	PATENT ABSTRACTS OF JAPAN vol. 016, no. 249 (C-0948), 8 June 1992 (1992-06-08) -& JP 04 057853 A (CHLORINE ENG CORP LTD;OTHERS: 01), 25 February 1992 (1992-02-25) abstract	25
Y		26
Y	EP 0 732 378 A (NIPPON OIL CO LTD) 18 September 1996 (1996-09-18) column 1, line 37 - line 47 column 4, line 49 - line 53	26
X	DATABASE WPI Section Ch, Week 197633 Derwent Publications Ltd., London, GB; Class D13, AN 1976-62648X XP002129263 -& JP 51 076467 A (KYOWA HAKKO KOGYO), 2 July 1976 (1976-07-02) abstract	25,26
X	CHEMICAL ABSTRACTS, vol. 98, no. 21, 23 May 1983 (1983-05-23) Columbus, Ohio, US; abstract no. 177859, RAA, JAN ET AL.: "Isolation of astaxanthin from crayfish or shrimp waste for use as a coloring agent in fish feed" page 531; column 2; XP002129261 abstract -& NO 147 365 B 20 December 1982 (1982-12-20)	25

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 99/00987

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 009, no. 059 (C-270), 15 March 1985 (1985-03-15) -& JP 59 196032 A (FUMIO NISHIKAWA;OTHERS: 02), 7 November 1984 (1984-11-07) abstract	27

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

AKER877ITC00058055

RX-0135.0150

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

DUBUC, Jean H.
GOUDREAU GAGE DUBUC
The Stock Exchange Tower
800 Place Victoria, Suite 3400
P.O. Box 242
Montréal, Québec H4Z 1E9
CANADA

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	11.08.2000	
Applicant's or agent's file reference: CG/10857.274	**REPLY DUE**	**within 3 month(s)** from the above date of mailing
International application No.: PCT/CA99/00987	International filing date *(day/month/year)*: 21/10/1999	Priority date *(day/month/year)*: 21/10/1998
International Patent Classification (IPC) or both national classification and IPC: C11B1/10		
Applicant: UNIVERSITE DE SHERBROOKE et al.		

1. This written opinion is the first drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain document cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

   When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

   How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

   Also: For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

   If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 21/02/2001.

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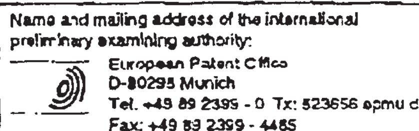
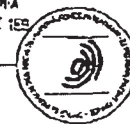
397-7602

Name and mailing address of the international preliminary examining authority:	Authorized officer / Examiner
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Rouault, Y
	Formalities officer (incl. extension of time limits) Mastropietro, M Telephone No. +49 89 2399 8092





Form PCT/IPEA/408 (cover sheet) (January 1994)

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-28 as originally filed

Claims, No.:

1-27 as originally filed

Drawings, sheets:

1/20-20/20 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	15,25
Inventive step (IS)	Claims	1-27
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Form PCT/IPEA/408 (Boxes I-VIII. Sheet 2) (January 1994)

WRITTEN OPINION
SEPARATE SHEET

International application No. PCT/CA99/00987

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: JP-A-53112195
D2: Chem. Abs. 177859

Novelty (Art. 33(2) PCT)

Claims 15 and 25 are not novel.
Document D1 describes a method to extract fat from, e.g. Horse Mackerel by placing said animal preferably in acetone followed by drying at 40-60 °C. The step of separating the liquid and solid content before drying is obvious.
The subject matter of claim 15 is therefor not new.
Document D2 discloses a shrimp extract (krill is a shrimp) containing 445 mg/L of carotenoïd in astaxanthin. The krill extract is less than 200 kg, taking into account that the density of soybean oil is less than the density of water. Hence, the krill lipid extract carotenoid in astaxanthin is at least 220µg/g of krill extract.
The subject matter of claim 25 is therefor not new.

Inventiveness (Art 33(3) PCT)

The present set of claims is not based on an inventive step. The proposed method of extraction does not seem to improve the amount of extracted products. The results are often worst than when using the method of Folch et al. (see Table 7 to 11).
P.9 l. 29-30, the applicant writes that the lipids extracted with the Folch method are toxic. No documents are cited to support this affirmation. Also, the applicant has not shown a particular selectivity of his method over the method of Folch (see Table 12).

Because of the absence of any quantitative or qualitative technical effect, neither the objective problem nor the solution to this problem can be defined. The present application cannot be considered as involving an inventive step (Article 33(3) PCT).

WRITTEN OPINION
SEPARATE SHEET

International application No. PCT/CA99/00987

Re Item VII

Certain defects in the international application

The symbol ":" in claim 25 is obviously an error and has been interpreted as "μ".

Re Item VIII

Certain observations on the international application

Claims 25 and 26 are not supported by the description as required by Article 6 PCT, as their scope is broader than justified by the description. The reason therefor is the following: the amount of carotenoid in asthaxanthin or in canthaxanthin per weight of krill extract has not been mentioned in the description or in the examples.

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THE FREEZE-DRYING TECHNOLOGY

AND THE LYO-TECH FREEZE DRIERS

General Information

MANUFACTURIER DE PRODUITS LYOPHILISÉS (SÉCHÉS À FROID)
FREEZE-DRIED PRODUCTS MANUFACTURER

LYO-SAN INC.
500 Aéroparc, C P 598
Lachute, Québec, Canada
J8H 4G4

Tél.: (450) 562-8525 e-mail: celine@lyo-san.ca
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FAX

À:	**Neptune technologie & bioressources**	De:	**Céline St-Pierre**	(Poste 28)
A/S:	**M. Roger Corriveau**		**Directrice générale**	
Fax:	(450) 972-6351			

Date: 12 avril 2002 Nombre total de pages incluant celle-ci: 12

Sujet : Document sur lyophilisation et lyophilisateur de Lyo-San.

Bonjour M. Corriveau,

Tel que convenu lors de notre conversation téléphonique de ce matin, vous trouverez ci-joint les documents suivants :

- the freeze-drying technology and the Lyo-Tech freeze driers
- Specifications , Lyo-Tech freeze drier Y5 model.

Vous pouvez également aller sur le site de l'ACIA (Agence canadienne de l'inspection des aliments), les noms des entreprises approuvées sont inscrits.

Voici le site internet : www.cfia-acia.agr.ca
Veuillez noter qu'il y a beaucoup d'information sur ce site et ce n'est pas toujours évident de trouver rapidement ce que l'on veut. Je vais vous envoyer un message par courriel qui vous donne le lien direct pour arriver sur la section qui vous permettra de voir le nom de Lyo-San dans les compagnies approvées pour les États-Unis et l'Union Européenne.

J'espère que ces informations vont vous aider pour votre dossier. N'hésitez pas à me contacter si des renseignements complémentaires sont nécessaires.

Recevez, M. Corriveau, mes salutations distinguées.

Céline St-Pierre

What is freeze-drying exactly?

Freeze-drying is a process by which we bring a product to a stage where it will contain a very low content of residual moisture. Water extraction from the product will assure a much better stability and conservation on a long term basis will then be possible.

Freeze-drying is using a simple physical transformation. This transformation is called sublimation and occurs when ice changes to water vapor directly by-passing the liquid phase so there is no presence of water at any time in the process.

To achieve freeze-drying, four conditions need to be met:

- the product has to be frozen
- the environment has to be under a negative pressure (vacuum)
- an energy source for sublimation must be available
- a water vapor trap must be available

Lyo-Tech freeze-driers are equipped to realize all those conditions efficiently. Following is a short description of the different equipment and explanation of their individual functions.

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Description and function of freeze-drier components

Sublimation chamber:	Cylindrical tank in which takes place the sublimation. When the door is closed the tank is airtight and can be put under vacuum.
Trays:	Recipient on which the product to be dried is placed.
Shelves:	Surface on which we put the trays in the sublimation chamber. The shelves may be cooled to freeze the product and heated to dry it.
Condenser:	Heat exchanger, placed inside the sublimation chamber, that can be cooled to recuperate the water vapor after sublimation.
Compressor:	Mechanical unit that achieves the cooling of the shelves and condensers.

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Vacuum pump:	Mechanical unit that achieves the vacuum inside the sublimation chamber.
Circulating pump:	Mechanical unit that takes the heating fluid and sends it to the shelves. By heating that fluid we can increase the temperature of the shelves and thus supply the necessary energy for sublimation.
Heating elements:	Immersed electrical elements that heat the heating fluid.
Control panel:	Panel on which are all control and instruments of the freeze-drier.

Why is freeze-drying the best?

Freeze-drying has numerous advantages if compared to other types of drying and conservation process.

1- Freeze-dried products can be stored at room temperature when packed in airtight containers. Transportation and storage cost are thus lower.

2- Freeze-drying produces an important weight loss that facilitates the handling. As an example, some foods can lose as much as 90 % of their initial weight.

3- The freeze-drying process will achieve drying at low temperature. The product is thus protected against overheating that will alter its quality.

4- Once reconstitued the freeze-dried product will recover its initial color, flavor and texture in a few minutes.

5- One of the most important advantages of freeze-drying is that this process can dry foods while keeping their vitamins, minerals and protein almost intact. Vitamins and protein are very sensitive to heat. Low temperature involved in this process will prevent deterioration of those important elements. Also since the drying is done under vacuum this will prevent oxydation of the vitamins.

The freeze-drying cycle

1-	Food preparation:	Foods are processed and trays are filled with the product.
2-	Freezing:	Trays are placed in the sublimation chamber, compressor is started, shelves begin to cool down and the product is freezing slowly.
3-	Vacuum:	Vacuum pump is started and pressure in the sublimation chamber drops.
4-	Sublimation:	Heat is switched on and drying starts.
5-	Opening:	Freeze-drier is opened and product is removed from the trays.
6-	Defrosting:	While the empty trays are sent to preparation, freeze-drier is defrosted and cleaned.

The normal cycle time for a freeze-drier is 24 hours.

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Internet : www.lyo-san.ca

SPECIFICATIONS

LYO-TECH FREEZE DRIER

Y5 MODEL

Equipement and accessories list

- One compressor
- One vacuum pump
- Two heating elements
- Two circulating pumps
- Three condensers
- One automatic defrosting system
- One control panel with electromechanical switch
- One Mcleod gauge for lecture of vacuum
- One digital temperature control

Technical information

Freeze-drier type:	trays for bulk products interior condensers wall mounted
Capacity:	from 66 to 88 kg of wet product per cycle depending on product to dry.
Total sublimation surface:	12.25m
Refrigeration system:	5.6KW compressor, using freon R502, water cooled. * minimum water consumption: 1100 l / hr * maximum water consumption: 1900 l / hr * water at 20C
Condensing system:	3 condensers total capacity of 85 kg of water minimum temperature: -65C
Vacuum system:	0.75KW vacuum pump pumping capacity of 500 l / min supplying a vacuum of 100 microns in 60 minutes approximately.

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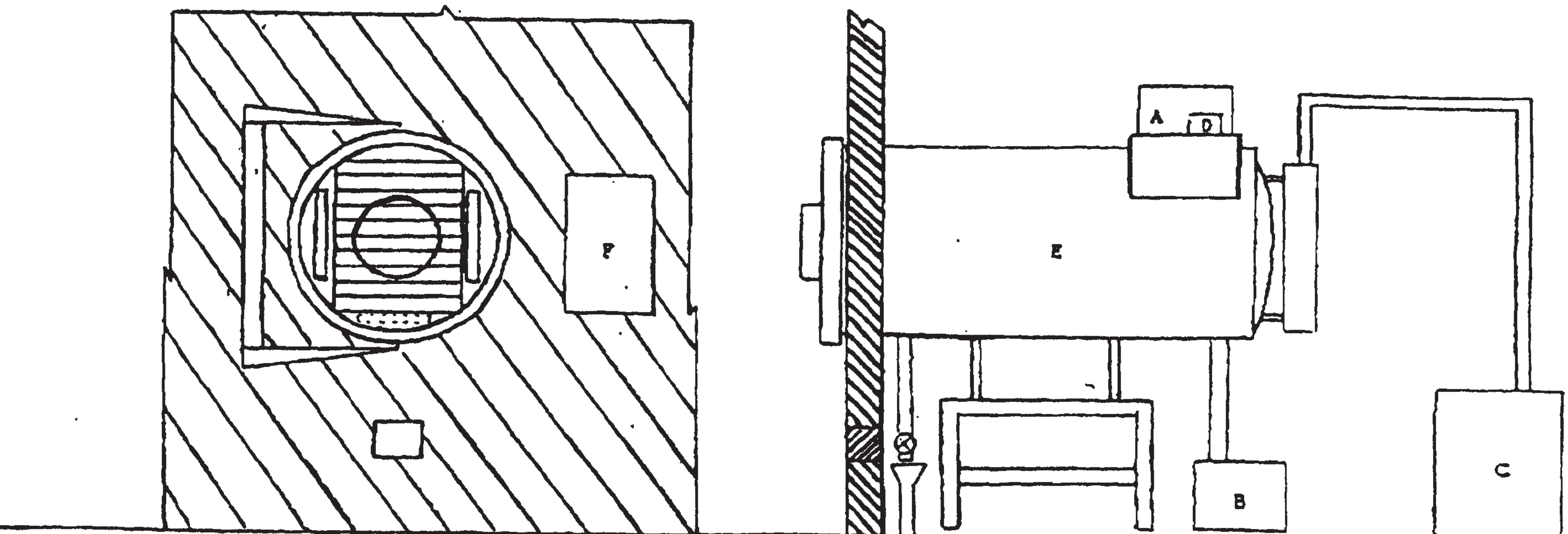
Heating system:	2 electrical elements of 6KW each
Quantity of trays:	44 (45 cm X 60 cm) stainless steel
Quantity of shelves:	11
* Space required:	2.5m deep 2.8m wide 2.5m high
Maximum power required:	22.8KW
Total electricity consumption per cycle: (approx.)	300KWh
Voltage:	240 Volts, one phase, 60Hz 600 Volts, three phase, 60Hz

* Could vary depending on layout.

LYO-TECH FREEZE-DRIER

Y5 MODEL



A: Heating fluid tank
B: Vacuum pump
C: Compressor
D: Circulating pump
E: Sublimation chamber
F: Control panel

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Workshop on the Essentiality of and Dietary Reference Intakes (DRIs) for

Omega-6 and Omega-3 Fatty Acids

ω6:ω3

Program and Abstracts

The Cloisters

National Institutes of Health

Bethesda, Maryland, USA

April 7-9, 1999

Sponsored by:

National Institute on Alcohol Abuse and Alcoholism-NIH

Office of Dietary Supplements-NIH

The Center for Genetics, Nutrition and Health

International Society for the Study of Fatty Acids and Lipids

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National Institute of Child Health and Human Development

Workshop on the Essentiality of and Dietary Reference Intakes (DRIs) for Omega-6 and Omega-3 Fatty Acids, The Cloisters, National Institutes of Health, Bethesda, MD, USA

April 7-9, 1999

Background

Following the 3rd Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) in Lyon, France, June 1-5, 1998, the ISSFAL Board of Directors agreed to convene a workshop on the essentiality of and DRIs for omega-6 and omega-3 fatty acids. An international group of experts will present reviews and new data in a round table format with ample time left for discussion. The participants will include speakers and discussants from the National Institutes of Health, other government agencies, academia, industry, non-profit organizations, the World Health Organization, the Food and Agriculture Organization, and the Food and Nutrition Board.

Venue

The Workshop will be held in the Mary Woodard Lasker Center for Health Research & Education (The Cloisters, Building 60) at the National Institutes of Health in Bethesda, Maryland, USA.

Conference Secretariat

The Center for Genetics, Nutrition and Health, 2001 S Street, NW, Suite 530, Washington, D.C. 20009 USA, phone: (202) 462-5062, fax: (202) 462-5241, e-mail: cgnh@bellatlantic.net.

Hotel Accommodations

We have selected *The Bethesda Ramada* in Bethesda, Maryland, as our hotel [8400 Wisconsin Avenue, phone: (301) 654-1000], since it is within walking distance of the National Institutes of Health (NIH). Parking on the campus of the NIH is very limited. The *Medical Center* Metro stop (Red Line) is on the NIH campus.

Conference Cochairs

Artemis P. Simopoulos, M.D. (USA)

Norman Salem, Jr., Ph.D. (USA)

Alexander Leaf, M.D. (USA)

Sponsors

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Roche Vitamins, Inc.

Workshop on the Essentiality of and Dietary Reference Intakes (DRIs) for Omega-6 and Omega-3 Fatty Acids

National Institutes of Health, The Cloisters

April 7-9, 1999

WEDNESDAY, APRIL 7, 1999

Welcoming Remarks - Enoch Gordis, M.D., NIAAA-NIH

Claudio Galli, M.D., President, ISSFAL

Session I. Principles to be Considered in Determining Essentiality and DRIs

Cochairs: Artemis P. Simopoulos, M.D.

Harald S. Hansen, Ph.D., D.Sc.

9:00 - 9:30 a.m. *Criteria for Determining Essentiality and Standards for DRIs*

Vernon R. Young, Ph.D., D.Sc.

9:30 - 10:00 a.m. *Essentiality of Omega-3 Fatty Acids*

Arthur A. Spector, M.D.

10:00 - 10:30 a.m. *Defining the Omega-3 Status in Mammals*

Andrew J. Sinclair, Ph.D.

10:30 - 11:00 a.m. Coffee Break

11:00 - 11:30 a.m. *An Evolutionary View of Diet Recommendations*

S. Boyd Eaton, M.D.

11:30 - 12:30 p.m. Discussant and General Discussion

Harald S. Hansen, Ph.D., D.Sc.

12:30 - 2:00 p.m. Lunch

Session II: Essential Fatty Acids and Central Nervous System Function

Cochairs: Norman Salem, Jr., Ph.D.

William C. Heird, M.D.

2:00 - 2:30 p.m. *Evidence for the Essential Nature of DHA for the Human and Rat Nervous System*

Norman Salem, Jr., Ph.D.

2:30 - 3:00 p.m. *DHA Supplementation of Breastfeeding Mothers: Effects on Maternal Plasma and Milk Fatty Acids, Infant Plasma Fatty Acids, Infant Visual*

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Function and Infant Neurodevelopmental Status

William C. Heird, M.D.

3:00 - 3:30 p.m. *Functional Basis for the Importance of Omega-3 Fatty Acids in Retinal and CNS Development*

Martha Neuringer, Ph.D.

3:30 - 4:00 p.m. *Long Chain Polyunsaturates and Human Visual Development*

Eileen Birch, Ph.D.

4:00 - 4:30 p.m. Coffee Break

4:30 - 5:00 p.m. *The Effects of DHA on Hostility*

Tomohito Hamazaki, M.D., Ph.D.

5:00 - 5:30 p.m. *Omega-3 Fatty Acids in Mood Disorders*

Andrew L. Stoll, M.D.

5:30 - 6:30 p.m. Discussants and General Discussion

Peter Willatts, Ph.D.

Joseph Hibbeln, M.D.

7:30 - 10:00 p.m. Dinner at the Bethesda Ramada

THURSDAY, APRIL 8, 1999

Session III. Cardiovascular Disease

Cochairs: Alexander Leaf, M.D.

Raffaele De Caterina, M.D., Ph.D.

9:00 - 10:00 a.m. *Polyunsaturated Fatty Acids and Cardiovascular Disease*

Alexander Leaf, M.D.

10:00 - 10:30 a.m. *n-3 Polynsaturated Fatty Acids Inhibit COX-2 Expression*

Raffaele De Caterina, M.D., Ph.D.

10:30 - 11:00 a.m. Coffee Break

11:00 - 11:30 a.m. *Alpha-Linolenic Acid in the Prevention of Cardiovascular Disease*

Serge Renaud, M.D.

11:30 - 12:00 p.m. *Omega-3 Long Chain PUFA and Triglyceride Lowering: Minimum Effective Intakes*

William S. Harris, Ph.D.

12:00 - 12:30 p.m. *Efficacy of n-3 PUFA and vitamin E in 11,324 post-MI patients: Results of GISSI-PREVENZIONE*

Roberto Marchioli, M.D.

12:30 - 1:00 p.m. Discussant and General Discussion

William E. Lands, Ph.D.

1:00 - 2:00 p.m. Lunch

Session IV: Relationship of Essential Fatty Acids to Saturated, Monounsaturated, and Trans Fatty Acids

Cochairs: Claudio Galli, M.D.

Andrew J. Sinclair, Ph.D.

2:00 - 2:30 p.m. *Relationships Between Saturated, Monounsaturated, Polyunsaturated Fatty Acids: Dietary Data vs. Data from Plasma Fatty Acid and Lipid Analyses*

Claudio Galli, M.D.

2:30 - 3:00 p.m. *Nutritional and Metabolic Interrelationships Between Omega-3*

Fatty Acids and Trans Fatty Acids

Bruce J. Holub, Ph.D.

3:00 - 3:30 p.m. Coffee Break

3:30 - 4:00 p.m. *Choice of Monounsaturated, Trans and Omega-3 Fatty Acid-Rich Oils*

for the Prevention of Excessive Linoleic Acid Syndrome

Harumi Okuyama, M.D.

4:00 - 5:00 p.m. Discussion

FRIDAY, APRIL 9, 1999

Session V. Dietary Recommendations and Omega-6:Omega-3 Ratio (LA, LNA, AA, EPA, DHA)

Cochairs: Peter R.C. Howe, Ph.D.

Bruce J. Holub, Ph.D.

9:00 - 9:20 a.m. *Intakes of Dietary Fatty Acid in the United States: Results from the USDA's 1994-1996 Continuing Survey of Food Intakes by Individuals*

Gary J. Nelson, Ph.D.

9:20 - 9:30 a.m. *World Health Organization/Pan American Health Organization*

(Status of EFA Worldwide)

Manuel Peña, M.D.

9:30 - 9:40 a.m. *n-3 Fatty Acids: Food Supply, Food Composition*

and Food Consumption Data

William D. Clay, Ph.D.

9:40 - 9:52 a.m. *BASF's Approach to Commercialization of Long Chain*

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Omega-3 Fatty Acids

Herbert D. Woolf, Ph.D.

9:52 - 10:04 a.m. *Essential Fatty Acids and the Products of the Groupe Danone for Human Nutrition*

Dominique Lanzmann-Petithory, M.D.

10:04 - 10:16 a.m. *Advantages and Disadvantages of the Use of Flax Seed as a Source of Omega-3*

Paul A. Stitt, Ph.D.

10:16 - 10:28 a.m. *Omega-3 LC-PUFA ñ from a Health Concept to Foods in the Shelves*

Reto Muggli, Ph.D.

10:28 - 10:40 a.m. *Infant Formulas with no DHA or ARA.. Are They Causing Harm?*

David J. Kyle, Ph.D.

10:40 - 11:00 a.m. Coffee Break

11:00 - 11:12 a.m. *Clinical Safety Studies of LCPUFA Supplementation of Premature and Term Infant Formulas*

James W. Hansen, M.D., Ph.D.

11:12 - 11:24 a.m. *Omega-3 Long Chain PUFA ñ Closing the Nutritional Gap*

Jacques Boudreau

11:24 - 11:36 a.m. *OmegaTech, Inc.*

William R. Barclay, Ph.D.

11:36 - 11:48 a.m. *Safety of Omega-3 Products Based on Fish Oil as Starting Material*

Bjorn Rene

11:48 - 12:00 p.m. Other

12:00 - 1:00 p.m. Discussants and General Discussion

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Bruce Holub, Ph.D.

Rebecca Costello, Ph.D.

1:00 - 2:00 p.m. Lunch

Session VII. Conclusions and Recommendations

Cochairs: Alexander Leaf, M.D.

Artemis P. Simopoulos, M.D.

2:00 - 5:00 p.m. *Roundtable Discussion*

ABSTRACTS

Wednesday, April 7, 1999

Session I. Principles to be Considered in Determining Essentiality and DRIs

Criteria for Determining Essentiality and Standards for DRIs

Vernon R Young, Ph.D., D.Sc.

Massachusetts Institute of Technology, Cambridge, MA 02139, USA

This introductory presentation to the workshop will begin with an initial, brief statement about the importance of knowledge on the quantitative needs for nutrients and the multiple uses of nutrient-based dietary reference values. From this introduction we will turn to (i) a consideration of the evolving conceptual and factual basis underlying the "essentiality" of nutrients and (ii) the definition and description of dietary reference intakes (DRIs). The latter include (following the structure proposed and applied recently by the US Food and Nutrition Board/Institute of Medicine/National Academy of Sciences):- Estimated Average Requirement (EAR); Recommended Dietary Allowance (RDA); Adequate Intake(AI) and Upper Tolerable Level (UL). The most useful DRI is the EAR, the reasons for which will be examined. Then a detailed discussion will follow with respect to the establishment of DRIs, including an emphasis on (a) the choice of the criterion (criteria) of nutrient adequacy chosen to establish a specific DRI and (b) the approach(es) that might be taken and data that are desirable to achieve this goal. The importance of seeking a congruence of evidence, where this is possible, in arriving at a DRI will be emphasized, by example. Finally some suggestions will be made with respect to the setting of DRIs for omega-3 and omega-6 fatty acids.

Essentiality of Omega-3 Fatty Acids

Arthur A. Spector, M.D.

Department of Biochemistry, University of Iowa College of Medicine,

Iowa City, Iowa 52242, USA

There is a growing consensus that omega-3 fatty acids are essential nutrients for humans. Much of the evidence is based on physiological measurements such as neurological development and visual acuity. To better understand why this class of polyunsaturated fatty acids is required, we must determine the biochemical basis for the essentiality. Of the eight fatty acids that comprise the omega-3 metabolic pathway, the two that are most likely to have essential biochemical functions are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

EPA can be converted to prostaglandins, thromboxanes and lipoxygenase products. However, no essential role for these EPA-metabolites has been reported, and it seems unlikely that the formation of these products is the reason that omega-3 fatty acids are essential. When elevated amounts of EPA are available, the incorporation of arachidonic acid (AA) into cell phospholipids and its conversion to eicosanoid mediators is reduced. Thus, EPA acts as a competitive inhibitor of AA, and this probably accounts for some of the beneficial effects of omega-3 fatty acids in the treatment of cardiovascular and inflammatory diseases. While the possibility that EPA is essential in order to modulate the effects of AA cannot be ruled out, the amounts ordinarily present in the plasma and tissues probably are too low to competitively inhibit the actions of AA. Therefore, modulation of AA metabolism is more likely to be a pharmacological effect of omega-3 fatty acid supplements rather than an essential physiological function.

The basis for considering DHA as the biochemically essential omega-3 component is much more compelling. DHA is the most abundant omega-3 fatty acid in most tissues, and it is present in large amounts in the brain and retina. DHA is the omega-3 fatty acid required for normal development of the nervous system and optimum visual acuity. Furthermore, when an omega-3 fatty acid deficiency exists, the body compensates by replacing it with the corresponding fatty acid of the omega-6 series, omega-6 docosapentaenoic acid (DPAn-6). These findings strongly suggest that DHA has an essential biochemical function. The most likely possibility is a membrane structural effect involving the packing of phospholipid head groups or the interaction of the lipid domains with membrane proteins. The lipids that contain the highest percentages of DHA are ethanolamine plasmalogen, phosphatidylethanolamine and phosphatidylserine. Therefore, it is likely that the function of DHA involves the metabolism, trafficking or physical properties of these phospholipids. Other possibilities that must be considered include the conversion of DHA to a lipid mediator, binding of DHA to a nuclear receptor that regulates gene expression, or formation of a DHA-centered free radical.

A central question concerning the essentiality of omega-3 fatty acids is why DHA rather than the corresponding member of the omega-6 series, DPAn-6, fulfills this purpose. The usual Western diet contains 10- to 20-times more omega-6 fatty acid, and the same metabolic pathway is utilized by both fatty acid classes. One possibility is that DHA is utilized more efficiently than DPAn-6.

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However, studies with neural cells in culture indicate that there is no appreciable difference in the uptake, retention or incorporation into phospholipids of DHA as compared with DPAn-6. While more detailed measurements may reveal a functional difference between DHA and DPAn-6, no such evidence is currently available. This suggests that DHA is utilized rather than DPAn-6 because it is more available to the tissues. Although the absolute amounts of these fatty acids in the plasma lipids are very small, there ordinarily is about five-times more DHA than DPAn-6. Furthermore, the main product formed by cultured astrocytes from omega-3 fatty acid precursors is DHA, whereas the main omega-6 product is AA. Astrocytes are the site where most of the polyunsaturated fatty acid precursors are elongated and desaturated in the brain. Thus, much more DHA than DPAn-6 appears to be available in the central nervous system.

These findings suggest the following hypothesis regarding the essentiality of omega-3 fatty acids.

Certain tissues, especially parts of the central nervous system, require a relatively large amount of a 22-carbon polyunsaturated fatty containing a 4,5-double bond for optimum function. The omega-6 metabolic pathway cannot satisfy this requirement because it operates primarily to produce AA for eicosanoid and inositol phospholipid synthesis. While some docosatetraenoic acid (22:4n-6) is made, it is primarily retroconverted to AA rather than proceeding down the pathway to form DPAn-6. Therefore, even though more omega-6 fatty acid precursors are available, the omega-6 pathway cannot produce enough DPAn-6 to satisfy tissue requirements. By contrast, the main product of the omega-3 pathway is DHA, not the 20-carbon intermediate. This fundamental difference in the operation of the polyunsaturated fatty acid metabolic pathway is likely to be the biochemical reason why omega-3 fatty acids are essential.

(Supported by NIH grants HL49264 and CA66081)

Defining the omega 3 status in mammals

AJ Sinclair, Ph.D.

Department of Food Science, RMIT University, Melbourne, Victoria, Australia 3001

This talk examines the status of omega 3 polyunsaturated fatty acids (PUFA) as essential nutrients in mammals. The first issue to be addressed is the importance of having a surrogate champion which promotes the cause of a nutrient on a daily basis. The question, ***what is your cholesterol level,*** is a message which sustains the cholesterol-heart disease story. Clearly, despite the importance of anti-oxidants, fibre, folate and anti-platelet therapy in CHD, cholesterol has been a survivor. Do the omega 3 PUFA have such a champion? In other words, do we have the data to support the importance of these essential nutrients; the existence of the MRFIT data and the more recent secondary prevention data from France and India provide strong support for the essentiality of the omega 3 PUFA.

The history of the EFA reveals that the omega 3 PUFA were ignored by most for 40 years or more.

Why is this so? If their dietary absence was associated with more obvious clinical symptoms, there is no doubt there might have been an omega 3 champion. The rather subtle effects of the dietary absence (or low intakes) makes it hard to sell to the general public. For example, we know that effects of deficiency on the electroretinogram (ERG) amount to a loss of a- and b-wave amplitudes of say 30% with perhaps other more substantial losses in sub-components, however we cannot yet say what this might mean in terms of "vision" which is what the public relate to. Perhaps, we too often ignore the fact that in the EFA field there are substitute fatty acids which prevent complete absences of say 22 carbon PUFA in the retina (e.g. 22:5n-6 or 22:3n-9 substitute for 22:6n-3 in omega 3 and EFA deficiency, respectively). This argues for the importance of these types of PUFA in this tissue, however necessarily the availability of such substitutes reduces the physiological impact of a dietary deficiency. Perhaps, we should be looking for a tissue where it is possible to alter the DHA content without the substitute PUFA being present. Such a tissue is the guinea pig heart - with an ALA rich diet the level of DHA is less than 1% of the phospholipid fatty acids and it is only on the inclusion of DHA that the heart DHA level rises. Given the sound data showing the crucial role of omega 3 PUFA and DHA, in particular, on cardiac function in other species/situations, surely this tissue in this species might be a useful research tool.

In the early years, linoleic acid had a prominent role as an anti-cholesterol fatty acid, however since the 1970ís the omega 3 PUFA have made a comeback in heart disease, vision and other diverse areas such as arthritis, bone development and neurological disorders. Where we currently stand is that we have much data on diet and the effect on tissue fatty acids, but relatively few data on exact intakes titrated against physiological function. This is especially true in the omega 3 and electroretinography field which was the first area where omega 3 PUFA (ALA!) were shown to have a specific physiological role. Furthermore, much of our research could be criticized because there are few studies where pure ALA has been used. It is surely no longer adequate to compare oil A (poor in ALA) with oil B (containing ALA) because of our ever increasing understanding of the potential actions of the many compounds found in the unsaponifiable fraction of

naturally-occurring oils. This highlights the need for pure ALA for research purposes.

Finally, we might be responsible for diluting the message of essentiality for the omega 3 PUFA because of the many ëargumentsí in house and in the public arena regarding the nutritional importance of ALA versus the long chain PUFA (EPA and DHA). I think it is instructive to recall that the research data show that ERG function is optimal in all animal models with dietary ALA and not dietary DHA and that the de Lorgeril and Singh data on secondary prevention indicate a role for ALA in the cardiac area.

An Evolutionary View of Dietary Recommendations

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Traditional Research - satisfactory for preventing classical deficiency

syndromes; less so for reducing chronic degenerative disease risk:

1. Clinical trials - generally focus on diagnosis or treatment, not prevention.

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2. Mechanistic studies - limitless possible study subjects; limited funding, facilities, and investigators.

3. Epidemiology - conflicting results emphasized by media leading to public confusion and skepticism. Examples:

a. vitamin E, β carotene and lung cancer

b. fat and coronary heart disease

c. fiber and colon cancer

d. salt and overall mortality

e. calcium and osteoporosis

f. fat and breast cancer

Needed: Additional Approach - to focus future efforts, reconcile past investigative discrepancies, and provide solid theoretical basis for entire field. Viewing nutrition from the perspective of human evolutionary experience might achieve these ends.

Evolution and Nutrition

1. Basic Premise - current humans are genetic Stone Agers; cultural change since agriculture has exceeded capacity of genetic evolution to keep pace.

2. Essential goal - determine character of human nutrition during Stone Age experience.

3. Investigative approaches:

a. analysis of recent forager subsistence patterns

b. analysis of human skeletal remains - gross anatomy and radioisotopic

c. archaeological finds - animal remains, botanical residues, implements

d. nutritional analyses of game animals and wild plant foods - similar to those available before agriculture

4. Modeling

$A(C^aX) + V(C^vX)$ = daily energy intake

A and V - mean energy content (kcal/g) of animal and vegetable foods

C^a and C^v - proportions of animal and vegetable foods, respectively

X - total number of grams of food required to provide daily energy

5. Previously reported results:

a. Protein: 30-35% total energy

b. Carbohydrate: 40-50%

c. Fat: 20-25%

Recently Revised Model Inputs

1. Hunter-gatherer subsistence patterns

a. old mean: 35% animal : 65% plant (by weight)

b. revised mean: 45% animal : 55% plant

2. Improved assessment of game nutritional properties

a. old view - based solely on muscle meats (i.e. "selected cuts")

b. new view - hunter-gatherers actually consume "total edible," hence fat content is 1.5 - 18%, not 1 - 5%.

New Estimates:

Mean macronutrient contribution (% total energy)

a. Protein 30-33%

b. Carbohydrate 31-34%

c. Fat 36%

General Fat Characteristics:

1. Energy contribution similar to Mediterranean (and current American) pattern; unlike East Asian paradigm.

2. But character of fat is much different from U.S. pattern:

Paleolithic U.S.

% saturated less more

% C18 more less

% C14 + C16 less more

% monounsaturated more less

% polyunsaturated more less

% C20 + C22 more less

ω6 : ω3 lower higher

New Inputs Alter Essential Fatty Acid Retrojections:

Paleolithic Current

1998 Estimate 1999 Estimate American

Total C20 + C22 3.01 g/d 5.79 g/d 0.80 g/d

AA : ω3 LCP 1.68 1.43 5.6

Overall ω6 : ω3 0.79 1.39 > 10.0

Wednesday, April 7, 1999

Session II. Essential Fatty Acids and Central Nervous System Function

Evidence for the Essential Nature of DHA in the Human and Rat Nervous System

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A series of experiments were performed which demonstrate that diets that are low in n-3 fatty acids lead to low brain DHA and also lead to losses in nervous system function. Diets were constructed that varied only in the amount of alpha-linolenic acid intake derived from flax oil and adequate in linoleic

acid derived from safflower oil. No long chain (20C or more) polyunsaturates were present in these diets. Rats were raised for three generations on these diets and animals were tested at adulthood in the second and third generation. Brain and retinal DHA was markedly depressed in the second and third generations with increases in the long chain n-6 polyunsaturates, especially docosapentaenoate (22:5n6). Accompanying this "reciprocal replacement" of DHA were significant losses in performance on behavioral tasks related to learning and memory. The n-3 deficient rats acquired an olfactory discrimination task more slowly and made significantly more errors. This was significant as it extends the constellation of deficits described in n-3 deficiency to another sensory modality in addition to vision. In addition, n-3 deficient rats showed delayed escape latency in the Morris Water Maze task. This was more pronounced in the third generation where the DHA deficit was slightly greater relative to the second generation. Motor activity was not significantly different between groups. The swimming speed and distance traveled was greater for the n-3 deficient animals, yet they took a longer time to find the platform. In a subsequent memory test with the platform removed, the deficient animals made fewer crossings of the former position of the platform indicating that the n-3 adequate group better retained the memory of the position of the platform. This effect was particularly pronounced in the third generation. These experiments show that there are functional deficits associated with low brain DHA that may relate to sensory function, but it is more likely that they are due to losses in higher level functions related to information processing in the brain that are necessary for memory and learning.

In the second series of experiments, the focus was on the level of alpha-linolenate necessary to support nervous system DHA levels. An artificial rearing system was used to control the EFA content of rat pup diet from day 5-18 of life. At weaning ratios of linoleate to alpha-linolenate of 10:1 and even 1:1 did not produce the same level of brain DHA as a 1:12 ratio or that of dam-reared pups whose mothers were fed a diet containing 1.1% DHA as well as other LCPs, i.e., were well nourished. However, the 1:12 ratio led to a decrease in brain AA while the 10:1 ratio led to a slight increase over the dam-reared level. There was a similar picture in the retina, with the exception that even the extreme case of LA/LNA of 1:12 did not support the same level of retinal DHA as that of dam-reared animals. The high LNA diet (1:12) again led to a significant decrease in retinal AA. Thus it appears that increasing the level of alpha-linolenic acid in developing mammals is not an entirely adequate solution to the problem of supporting the neural DHA at a level comparable to that of a well nourished maternal reared individual. Raising the n-3 content to a 1:1 level does support a balanced EFA composition of the nervous system to a much greater extent that the 10:1 ratio, a ratio that is more typical of human infant formulas in North America.

The third issue to be addressed is the applicability of these studies to humans. Essential fatty acid metabolism was assessed *in vivo* in adults and in infants of various gestational ages and birth weights. A controlled trial in adults demonstrated conclusively that linoleic acid is converted to arachidonate and alpha-linolenate is converted to DHA. The rates of the n-6 metabolism appear faster than the n-3 conversions, in contrast to some previous findings. Increased levels of n-3 fatty acids associated with a fish-poultry based diet led to decreases in deuterium incorporation in DHA. Smoking and alcohol intake were associated with increased deuterium incorporation into DHA from linolenate. Infants are capable of LA to AA and LNA to DHA conversion *in vivo* within the first week of life even when born very prematurely (e.g., 1 kg BW). In fact, it was surprising that there was an inverse correlation of deuterium enrichment of DHA with gestational age. Although it is clear that premature and term infants express EFA metabolic activity, it must be understood that these are trace level studies; the metabolic activity towards DHA in particular is very limited and unlikely to be adequate to support rapid brain and organ DHA accretion during the first months of life.

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Docosahexaenoic Acid (DHA) Supplementation of Breastfeeding Women: Effects on Maternal Plasma and Milk Fatty Acids, Infant Plasma Fatty Acids, Infant Visual and Neurodevelopmental Function and Indices of Maternal Depression

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DHA, an important component of the structural lipids of brain and retina, is present in human milk but not in formulas currently available in the United States and it has been suggested that the better visual and cognitive development of breastfed infants is due, at least in part, to the presence of DHA in human milk. However, the DHA content of the milk of U.S. women, which is dependent on maternal plasma lipid DHA and, hence, intake of α -linolenic acid and/or DHA, is less than that of many other populations. Further, the DHA content of maternal plasma lipids decreases during lactation. Thus, it has been suggested that breastfeeding women and their infants might benefit from maternal DHA supplementation. Indeed, we and others have shown that maternal DHA supplementation prevents the usual decline in maternal plasma lipid DHA content and increases the DHA content of maternal plasma as well as that of milk and the recipient infantsí plasma phospholipid. Based on these data, we hypothesized that maternal DHA supplementation also would result in better visual and neurodevelopmental status of the recipient infants and lessen the incidence of maternal depression which, in epidemiological studies appears to be higher in populations with low DHA intake.

To test these hypotheses, women were assigned randomly and blindly to receive either ~200 mg of DHA daily (n=80) or a placebo (n=65) for 120 days after delivery. Visual function of infants was assessed by transient visual evoked potentials (VEP) and visual acuity was measured by sweep VEP and the Teller Acuity Card Procedure at 4 and 8 months of age. Infant neurodevelopmental status at 12 months of age was assessed by the Clinical Adaptive Test/Clinical Linguistic and Auditory Milestone Scale (CAT/CLAMS) and the Gesell Gross Motor Developmental Quotient (GM DQ). Maternal depression was assessed by the Beck Depression Inventory (BDI), the Edinburgh Postnatal Depression Scale (EPDS) and the Structured Clinical Interview for Depression (SCID).

There were no differences at either 4 or 8 months in VEP latency, VEP amplitude, sweep VEP acuity or Teller acuity between groups whose mothers did or did not receive DHA. There also were no statistically significant differences in mean CAT (111.2 ± 11.0 *vs.* 107.3 ± 9.3) or CLAMS (101.5 ± 16.0 *vs.* 100.9 ± 13.9) scores of infants whose mothers did or did not receive DHA; however, the mean GM DQ of infants whose mothers received DHA was significantly greater than that of infants of mothers who did not (102.6 ± 13.3 *vs.* 95.2 ± 12.7; p=0.03). The incidence of postpartum depression as assessed by BDI, EPDS or SCID did not differ between groups and was lower than expected in both groups.

We conclude that maternal DHA supplementation maintains or increases the DHA content of maternal plasma lipid and increases the DHA content of both maternal milk and the lipids of infant plasma. However, in this study, these positive effects of maternal DHA supplementation were not

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accompanied by better visual function, visual-motor problem-solving ability or language development of the recipient infant and also did not affect the incidence of maternal depression. On the other hand, maternal DHA supplementation resulted in the recipient infants having somewhat better indices of motor development at 12 months of age. These data, therefore, do not support our hypothesis that maternal DHA supplementation improves visual and neurodevelopmental status of the recipient infant or lessens the incidence of maternal depression. They provide little support for the concept that breastfeeding mothers require supplemental DHA.

Functional Basis for the Importance of Omega-3 Fatty Acids

in Retinal and CNS Development

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Infants fed standard infant formulas lacking DHA have low blood and tissue levels of DHA compared with those receiving pre-formed DHA or human milk. Whether dietary intake of omega-3 fatty acids has a substantial impact on CNS levels depends on the infant's stage of development and prior nutritional status. Preterm infants, who are at an earlier stage of brain development and of DHA accretion, are at greater risk than term infants of failing to achieve normal DHA levels in the retina and nervous system. However, animal studies have shown that low tissue levels are rapidly corrected once a dietary supply becomes available and, once incorporated into neural tissue, DHA is tenaciously retained. Therefore CNS levels in older children or adults are unlikely to be altered significantly by low dietary intake of omega-3 fatty acids.

For the purpose of defining essentiality, the more important question is whether a difference in fatty acid status during development is related to functional deficits. The most consistent effects of omega-3 fatty acid deficiency and supplementation have been on measures of visual system function. In monkeys and in preterm human infants, diets low in DHA's precursor, alpha linolenic acid, lead to poorer development of both visual acuity and the electroretinogram, a measure of retinal physiology. Furthermore, supplementation with pre-formed dietary DHA has been associated with enhanced visual acuity development in most studies of preterm infants and in some, but not all, of term infants.

It is assumed that these effects are mediated by differences in the fatty acid composition, and particularly the DHA content, of retinal and neural membranes. However, the underlying mechanisms for these effects, and the critical site(s) for these effects within the nervous system, are not clearly understood. Changes in the electroretinogram, which specifically measures retinal function, are hypothesized to be the result of changes in the biophysical properties of photoreceptor outer segment membranes, the site for the absorption of photons and their transformation into neural signals. These membranes contain the body's highest levels of DHA. Differences in visual acuity development, on the other hand, may be due to changes within photoreceptor membranes, other elements within the retina, the central visual pathway, and/or the visual cortex. Possible mechanisms include alterations in the development of the fovea, changes in retinal sensitivity, or changes in the synaptic connectivity or activity of the visual cortex.

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Studies of DHA supplementation in human infants have reported differences in visual acuity primarily during the first few postnatal months and in one major study at one year of age. The longer-term implications of these differences in infant acuity still are unclear, due to the lack of studies with more extended follow-up.

However, it is known that restriction of visual input during early development can lead to lasting effects on visual function, so it will be important to examine this issue more closely.

Differences in visual development are of interest not only in their own right, but also because they may reflect a more general effect on neural, and perhaps cortical, maturation. Studies reporting an advantage in intellectual development in breast-fed compared with formula-fed infants have prompted speculation that the DHA present in breast milk is a critical factor. However, the difference in DHA content is confounded with many other compositional differences, as well as socioeconomic and parenting factors which are known to strongly influence intellectual development.

In monkey studies and in randomized human clinical trials, differences have consistently been found in one aspect of cognitive development, visual attention. In monkey infants fed low levels of alpha linolenic acid, and preterm human infants fed formulas without DHA, the duration of fixations to visual stimuli are prolonged compared to infants with higher DHA status. Developmental psychologists have interpreted increased look duration as indicating slower speed of processing the stimulus and encoding it into memory. It is also possible that this effect reflects a specific difficulty in shifting or disengaging attention, an ability which develops during the first postnatal year, or a difference in the intensity of the infants' responses to visual stimuli. This effect appears to be independent of effects on visual acuity, as the two outcomes are not correlated in either monkey or human infants. Longer look durations are moderately correlated with poorer achievement in later tests of cognitive development, including IQ tests at school age. Thus, as with the effects on visual acuity, the implications of this difference for later development are unclear but worthy of further study.

Both animal and human studies of the effects of omega-3 fatty acid status on behavioral development have focussed on possible changes in cognition and learning. Other aspects of behavior generally have not been examined but are of equal interest. There are good rationales to hypothesize effects of omega-3 fatty acid status on, for example, sleep and temperament. Preliminary findings in rhesus monkeys indicate changes in both sleep and responsiveness to environmental stimuli. Changes in eicosanoids or in neurotransmitter metabolism provide plausible mechanisms for such effects.

The range of functional effects of omega-3 fatty acid deficiency and supplementation during development and their impact on later vision, cognition and behavior are not completely understood, nor are the relationship of these effects to the dose of dietary DHA and the age and duration of dietary intervention. These issues can only be resolved by longer-term studies with a range of dietary treatments and functional outcomes.

Long Chain Polyunsaturates and Human Visual Development

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The Effects of DHA on Hostility

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Numbers of studies have indicated an association between Type A behavior pattern (TABP) and CHD. TABP is characterized by aggression, hostility , excessive competitive drive, and time urgency. Because TABP is a vague and complex mixture of behavior patterns, many researchers began to investigate components of the TABP construct. Hostility is the most popular factor among them. Actually it better predicts important adult diseases than TABP.

We have been investigating the effects of DHA on extraggression (aggression against others, EA) of students using P-F study originally created by Rosenzweig. In P-F study testees are asked to give comments to frustrating pictures. Those comments are judged if they are aggressive against others (EA), self or nobody. EA contains three categories: obstacle-dominance, ego-defense (extrapunitive) and need-persistence. Comments are judged as ego-defensive, if comments contain hostile words to others, or aggressive denial or rejection against othersí reproach or accusation. Thus, we regarded ego-defense as hostility in the following studies.

According to our previous three-month double-blind study, hostility was enhanced by final exams in control students, whose average intakes of DHA were about 200 mg/d, whereas hostility was not enhanced in students who took DHA capsules (1.5-1.8 g DHA/d); in another study we found that if there was no stressor like exams, hostility was not changed significantly in either the control or the DHA group. We also found that DHA administration significantly enhanced the ratio of plasma epinephrine to norepinephrine in the DHA group compared with the control group during continuous psychological stress (final exams for two months). This DHA effect was mainly due to norepinephrine reduction in the DHA group.

Those studies above were all done with young adults. But people over 50 are more susceptible to stress-related adult diseases. Consequently, we decided to perform a similar study with older subjects to investigate the effects of DHA on hostility.

Method. Twenty-two males and 18 females of 50-60 yr of age volunteered for the present

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double-bind study. They were all healthy, and one half of them were farmers from suburban farming villages in Nakornpathom, Thailand. They were randomly allocated either to the DHA group (11 males and 8 females) or to the control group (11 males and 10 females). Subjects in the DHA group took 10 DHA capsules/d containing 1.5 g DHA as a total for two months, and those in the control group took 10 control capsules/d, each capsule containing 280 mg of mixed plant oil (47 % olive oil, 25 % rapeseed oil, 25 % soybean oil and 3 % fish oil). At the start and the end of the study, volunteers took P-F study. Just before they took P-F study at the end of the study, they watched a provoking videotape for 20 min as stressor. The videotape contained many cruel scenes from the real crimes and disasters.

Results. EA was significantly decreased in the DHA group (32 ± 15 % to 25 ± 11%, M ± SD, $p<0.02$), whereas not in the control group (27 ± 16% to 23 ± 10%). Inter-group difference was not significant by ANOVA. Hostility was significantly decreased in the DHA group (17 ± 8% to 11 ± 7%, $p<0.05$), whereas not in the control group (16 ± 11% to 12 ± 8%). Although the inter-group difference was not significant by ANOVA, the ratio of increment in hostility in the DHA group (2out of 19) was significantly ($p<0.05$) lower than in the control group (8 out of 21).

Discussion. We provoked subjects of both groups by videotape, but extra aggression or hostility did not increase in either group. The place where PF study was performed (Silpakorn University,

Nakornpathom) was not familiar to most of the volunteers. Consequently, there might be effects of becoming accustomed to the test in a very unfamiliar place at the end of the study. Although the effects were marginal compared with the case of young adults with natural stressor, it is likely that DHA influenced hostility of people even in their fifties. Taken into account that hostility is a risk factor of adult diseases, enough amounts of DHA (up to 1.5 g/d) might be beneficial.

Omega-3 Fatty Acids in Mood Disorders

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Omega-3 fatty acids may inhibit neuronal signal transduction pathways in a manner similar to lithium and valproate, two effective treatments for bipolar disorder. To examine this pharmacological similarity more closely, a study was performed to examine whether omega-3 fatty acids also exhibit mood-stabilizing properties in bipolar disorder. This was a 4-month, double-blind, placebo-controlled study, comparing omega-3 fatty acids (9.6 g/d) vs. placebo (olive oil), in addition to usual treatment, in 30 patients with bipolar disorder.

The results of the study revealed strong mood stabilizing and antidepressant effects of the omega-3 fatty acids. A Kaplan-Meier survival analysis of the cohort revealed that the omega-3 fatty acid patient group had a significantly longer period of remission than the placebo group ($p = 0.002$; Mantel-Cox). In addition, for nearly every other outcome measure, the omega-3 fatty acid group performed better than the placebo group. Omega-3 fatty acids were well-tolerated and improved the short-term course of illness in this preliminary study of patients with bipolar disorder.

The omega-3 fatty acids offer some unique benefits, should they prove to be truly effective mood stabilizers. The advantages of the omega-3 fatty acids as mood stabilizers include the apparent acute efficacy in both the manic and depressive phases of bipolar disorder, their lack of toxicity, as well as high patient acceptance. In addition, omega-3 fatty acids confer some health benefits during chronic use, such as possible reduction in the risk of a fatal myocardial infarction. In addition, the omega-3 fatty acids have no documented adverse drug interactions, and appear to be safe (and possibly beneficial) in pregnancy and in children.

The disadvantages of the omega-3 fatty acids include their low potency, which results in a relatively large number of capsules per day. This may effect compliance. In addition, at the high doses used in the pilot study, several patients treated with either olive oil placebo or omega-3 fatty acids developed mild gastrointestinal distress, generally loose stools. This was completely abolished by lowering the dosage slightly or dividing the dosage into 3 or 4 separate portions. There is also the theoretical risk of increased bleeding during high-dose omega-3 fatty acid treatment. However, no change was observed in bleeding times during the controlled trial in bipolar disorder.

We have also treated more than 20 bipolar patients with open-label flaxseed oil. Flaxseed oil contains alpha-linolenic acid, a shorter chain omega-3 fatty acid. Measuring the clinical response to an open-label treatment is unavoidably subjective. However, the majority of the bipolar patients treated with flaxseed oil appeared to benefit. Many of these patients have described a distinct mood elevating effect from the flaxseed oil, and most have elected to remain on the flaxseed oil for the long-term. As with fish oil, the flaxseed oil was used adjunctively, in that the flaxseed oil was added to whatever mood stabilizing medication the patient was already receiving. The flaxseed oil was generally better tolerated than fish oil. However, whether causally related or not, we have observed several cases of hypomania in bipolar patients treated with flaxseed oil.

Our results support other data suggesting that the mechanism of action of mood stabilizers in bipolar disorder is the suppression of aberrant signal transduction and inhibition of kindling processes. This is consistent with a model of abnormal signal transduction in the pathophysiology of bipolar disorder. If further studies confirm their efficacy in bipolar disorder, omega-3 fatty acids may represent a new class of membrane-active psychotropic compounds, and may herald the advent of a new class of rationally designed mood stabilizing drugs.

Thursday, April 8, 1999

Session III. Cardiovascular Disease

Polyunsaturated Fatty Acids and Cardiovascular Disease

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Coronary heart disease is the leading cause of death in the United States and in Western industrialized

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countries. Many reports have appeared since the epidemiologic evidence of Bang and Dyerberg called attention to the low mortality from coronary heart diseases (CHD) among the Greenland Eskimos, which they attributed to potential antiatherosclerotic effects of the diet high in oil of marine vertebrates. Many studies have documented the effects of fish oils on a number of biochemical and physiologic factors that are believed to affect the atherosclerotic process. There are also a considerable number of experimental studies in animals which show a reduction in atherosclerosis when diets high in saturated fatty acids and cholesterol are supplemented with fish oils. Notably among these are the beneficial effects reported in swine and in nonhuman primates, but even in a nonhuman primate negative results have been reported.

What Bang and Dyerberg noted among the Greenland Eskimos has been largely confirmed among the Japanese. The Zutphen study by Kromhout and associates and the reanalysis of the Multiple Risk Factor Intervention Trial by Dolecek showed an inverse relation between fish intake and mortality from CHD, as have other studies. Dolecek analyzed the larger Multiple Risk Factor Intervention Trial dividing the 6000 subjects in the control group (Usual Care) for that Trial into quintals according to their mean ingestion of n-3 polyunsaturated fatty acids from 0 to 0.66 g daily and found significant inverse correlations between the ingestion of these fish oils and coronary heart disease, all cardiovascular diseases and all-cause mortality with the highest quintal having lowest mortality rates of some 40 to 50%. However, the rapid atherosclerosis-like processes that often cause restenosis following coronary angioplasty are not prevented by dietary fish oil supplements. There has been one prospective, randomized, placebo-controlled, secondary clinical trial which has reported a 29% reduction in all cause and cardiovascular mortality at 2 years follow-up in patients advised to eat oily fish 2 to 3 times per week compared with those not so advised. Another secondary, single blinded, clinical trial reported a remarkable reduction in all cause mortality at 27 months mean follow-up and recently again at almost 4 year follow-up in the same cohort of some 70% compared to controls ñ The Lyon Heart Study ñ in which alpha-linolenic acid was considered the important dietary polyunsaturated fatty acid.

Reports by Charnock and McLennan have drawn attention to another aspect of coronary heart disease which the highly polyunsaturated fatty acids in fish oils seem to affect beneficially. They found that rats fed a diet high in a fish oil were protected from the fatal cardiac arrhythmias induced by experimental coronary artery ligation. We have confirmed their findings in dogs with Prof. George E. Billman, Ohio State University School of Medicine. We have then pursued the mechanism of the antiarrhythmic effect of the fish oil fatty acids. With isolated cultured heart cells we have produced arrhythmias with chemical agents which can cause fatal arrhythmias in humans. We have found in every instance that if we add the fish oil fatty acids to the fluid bathing the cells before we add the toxic agent, the arrhythmia is prevented. If we first induce the arrhythmia in the single cultured contracting heart cells and then add the fish oil fatty acids the arrhythmia is promptly stopped. This antiarrhythmic effect is due to stabilization of the excitability of every contracting cell in the heart. This in turn results from a modulating effect of the fatty acids on the ionic currents that initiate the heart beat. Further studies suggest strongly that the fatty acids interact with binding sites on the proteins of the ion channels thus affecting their conductivity to make the heart much less responsive to the electrical events that initiate fatal cardiac arrhythmias.

Once we had found that the polyunsaturated fatty acids modulate ion currents in an excitable tissue, the heart, we surmised that they must have a similar effect on all excitable tissues, since all utilize the same electrical communicating system ñ and they do! We have reported that in the brain (hippocampal CA1 neurons) the voltage dependent Na^{+} and the L-type Ca^{2+} currents are affected very much as are the same cardiac currents. One consequence of this action in the brain is that the

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electrical threshold for inducing generalized seizure activity in the rat using the cortical stimulation model, is increased. So these fatty acids are anticonvulsants as well as antiarrhythmic agents. With the findings by some psychiatrists that these same fatty acids are apparently beneficial in the management of depression and bipolar behavioral disorders, the finding of an important effect of the fatty acids on the electrical activity of brain cells may have broader health implications than just to cardiovascular diseases. There remains much to be learned; we are probably just scratching the surface of the importance of polyunsaturated fatty acids to health and the prevention of diseases.

N-3 Polyunsaturated Fatty Acids Inhibit COX-2 Expression

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N-3 polyunsaturated fatty acids (n-3 FA), including docosahexaenoic acid (DHA) exert anti-inflammatory and anti-atherogenic properties, mostly ascribed to competition with arachidonic acid (AA) as substrate for cyclooxygenases and 5-lipoxygenase. A cytokine-inducible cyclooxygenase (COX-2) expressed at sites of inflammation permits high production of prostanoids and amplification of the inflammatory response. We previously showed that n-3 FA (particularly DHA) are inhibitors of cytokine-induced expression of adhesion molecules in vascular endothelial cells (EC). Since genes for adhesion molecules and COX-2 share consensus sequences for transcription factors and patterns of cytokine induction, we hypothesized that n-3 FA might be transcriptional regulators of COX-2 expression. We therefore measured changes in AA metabolism in cultured human saphenous vein EC following 48 h preincubation with 25 µM DHA plus 24 h stimulation with IL-1 or LPS. We measured COX activity assessing 6-keto-PGF1a by RIA as a reflection of prostacyclin production. DHA decreased thrombin or AA-stimulated 6-keto-PGF1a to a greater extent in IL-1-stimulated EC than in the absence of IL-1, suggesting a greater inhibitory effect on COX-2 than on constitutively expressed COX-1. Inhibition of 6-keto-PGF1a production by DHA + the specific COX-2 inhibitor NS-398 was greater than inhibition by NS-398 alone, suggesting that DHA acted at a different level than on COX-2 enzymatic activity. Thus, COX-2 mRNA and protein expression were compared by Northern and Western analysis in control and DHA-treated EC stimulated with IL-1. DHA-treated EC showed a 50% inhibition of COX-2 expression at both mRNA and protein levels. Northern analysis of cells treated also with actinomycin D indicated that DHA exerted a transcriptional effect consistent with inhibition of NF-kB as assessed by electrophoretic mobility shift assays. These results show that treatment of EC with DHA reduces COX-2 protein expression and enzyme activity by transcriptional regulation likely to involve NF-kB activation, and offer a plausible alternative mechanism to many of the anti-inflammatory and anti-atherogenic effects of n-3 FA.

Alpha-Linolenic acid in the Prevention of Cardiovascular Diseases

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Cardiac mortality, especially sudden death, has been rarely prevented in dietary intervention trials to lower coronary heart disease (CHD). Only trials with an increased level of n-3 fatty acids (fish or fish oil) (DART Lancet 1989:2:757) have succeeded so far.

In Crete, cardiac death as shown by the seven country study is a rare event. In our duplication of the Cretan diet on 600 coronary patients (Lancet 1994:343:1454) cardiac death was reduced by 76 % and we did not observe any sudden death as compared to 8 in the control group with the prudent diet. Like the Crete population (Eur J Clin Nutr 1993:47:20), our subjects with the Cretan diet had a high level of oleic and alpha-linolenic acids in their plasma.

Studies have shown that arrhythmia of myocytes in culture, and ventricular fibrillation in dogs and rats are inhibited by n-3 fatty acids (Proc Natl Acad Sci USA 1997:94:4182). In rat reperfusion ventricular fibrillation was inhibited only by the alpha-linolenic acid rich canola oil but not by olive oil. (J Nutr 1995:125:1003)

In Crete it seems that it is through the consumption of walnuts, purslane and other greens as well as of snails, that a high intake of alpha-linolenic acid is achieved.

Recent prospective studies in USA (Harvard Public Health) and Europe (Euramic) indicate that the only fatty acid apparently inhibiting cardiac mortality in man is alpha-linolenic acid. Thus, alpha-linolenic acid, in addition to regulating the level of prostaglandins and leukotrienes, may be the chief fatty acid protecting from the CHD clinical manifestations, cardiac death and coronary thrombosis.

Omega-3 Long Chain PUFA and Triglyceride Lowering: Minimum Effective Intakes

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Author	Year	Source	ω3 FA (g/d)	Δ Trig*
Ågren [1]	1996	Fish	1.05	*-15% (PPL →)*
Schaefer [2]	1992	Fish	1.8	*-7% (PPL →)*
Silva [3]	1996	Shrimp	0.81	*-19%*

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Fahrer [4]	1991	Fish	1.75	***-19%***
Jacques [5]	1992	Fish	0.45	-8%
Gerhard [6]	1991	Fish	1.95	-1%
Brown [7]	1990	Fish	0.7	-7%
Ågren [8]	1988	Fish	0.8	***-16%***
Fehily [9]	1983	Fish	0.7	***-7%***
Brown [10]	1991	Capsules	1.5	***-25% (PPL ¬)***
Oosthuizen [11]	1994	Capsules	1.6	-17%
Valdini [12]	1990	Capsules	1.8	-16%
Gans [13]	1990	Capsules	1.8	***-33%***
Beil [14]	1991	Capsules	1.6	-20%
Radack [15]	1990	Capsules	1.1	-10%
Roche [16]	1996	Capsules	0.8	***-21% (PPL ¬)***
Demke [17]	1988	Capsules	1.5	-24%
Schindler [18]	1998	Capsules	1.1 (0.18 to 1.1 g/d)	***- 16-34%*** (depending phenotype)
Saldeen [19]	1998	Bread	0.3	***-17%***
Lovegrove [20]	1997	Multifoods	1.4	-4% (PPL ¬)
Sorensen [21]	1998	Margarine	0.9	***-12%***

*Bold italic = statistically significant. PPL = postprandial lipemia; ¬ = lower on ω3 FA

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Efficacy of n-3 PUFA and vitamin E in 11,324 post-MI patients:

Results of GISSI-Prevenzione

*Roberto Marchioli, M.D. on behalf of GISSI-Prevenzione Investigators.**

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The protective effects of fish oil supplements and vitamin E have been long debated.. Within Months of a myocardial infarction, 11.324 patients were randomized to an n-3 polyunsaturated fatty acid (PUFA) supplement (1g daily), a vitamin E supplement (300 mg daily), both, or neither. Baseline therapy included antiplatelet therapy in 90% of patients, beta blockers in 40%, and angiotensin converting enzyme inhibitors in 50%.

At 42 months follow up, patients who received n-3 PUFA had a significant 15% relative risk reduction in the combined rate of death plus nonfatal mycardial infarction and nonfatal stroke compared with those who did not receive n-3 PUFA (12.3% vs. 14.4% R= 0.001). By contrast, treatment with vitamin E caused a non significant 11% relative risk reduction in the combined endpoint. All of the beneficial effects of n-3 PUFA were due to 21% reduction in the risk of death.

There were no significant interactions between the two treatments. Both treatments were well tolerated. Gastrointestinal intolerance was the most commonly reported side effect.

* The GISSI-Group (Gruppo Italiano per lo Studio della Sopravvivenza nelliinfarto) is jointly sponsored by the Associazione Nazionale Medici Cardiologi Ospedalieri (ANMCO) and by Istituto Mario Negri Consorzio Mario Negri Sud.

Thursday, April 9, 1999

Session IV. Relationship of Essential Fatty Acids to Saturated,

Monounsaturated, and Trans Fatty Acids

Relationships Between Saturated, Monounsaturated, Polyunsaturated Fatty Acids : Dietary Data vs. Data from Plasma Fatty Acid and Lipid Analyses

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Background

Plasma levels of individual fatty acids (FA), of FA classes and of single intermediates in the metabolic series, are the result of diversified processes : intake with the diet, transport, uptake by cells and tissues, with the additional influence of metabolic processes (*de novo* synthesis or precursor - product conversion and retroconversion in the FA series , i.e. n-9, n-6, n-3).

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Determinants of plasma fatty acids

The relationships between major FA classes (saturates, SAT, monounsaturates, MUFA and polyunsaturated fatty acids, PUFA) in plasma lipids and the intake / synthesis from precursors, are rather different. In fact SAT and MUFA are synthesized *de novo*, besides being provided by the diet, whereas PUFA are exclusively supplied by the intake. In addition, long chain PUFA (LCP) of the n-6 and n-3 series in plasma and tissues represent a combination of amounts produced through the endogenous conversion of the short chain polyunsaturated fatty acids (SCP), linoleic (LA) and α - linolenic (ALA) acids to the LCP, and amounts provided directly by the diet. While the first pathway, i.e, synthesis from SCP, is the only source of LCP in strict vegetarians, the combined processes (intake + endogenous synthesis) take place in omnivorous subjects. It is however difficult to evaluate the relative contributions of these two components, due to the limited quantitative data on these "minor" individual LCP in foods. The assessment of LCP synthesis from SCP in individual subjects represents also a difficult task.

Additional factors which contribute to determine the final FA profiles in plasma lipids are : a. The different rates and degrees of esterification of individual FA into lipid classes (phospholipids, PL; cholesterol esters; CE; triglycerides,TG), as it emerges also from *in vitro* studies with cultured cells. b. The positional selectivity in the incorporation of different FA classes into glycerol. SAT are almost exclusively incorporated into the 1-position in cell PL and into the sn-1 and 3 position of TG, whereas MUFA are predominantly and PUFA almost exclusively incorporated into the 2-position. The 1-position is metabolically stable, whereas continuous replacement of FA takes place in the 2-position, through hydrolysis and reacylation processes. FA which in the 2-position should therefore be more readily modulated by changes in the relative availability of MUFA and PUFA. This should in turn result in a significant impact of the relative dietary intakes of these FA classes on the relationships between MUFA and PUFA in plasma lipids. In contrast, the intake of SAT should minimally affect their relative levels in circulating lipids.

Evaluations of FA relationships in plasma

We have measured several relationships between FA in plasma lipids, with the aim to establish possible correlations which could be of help in elucidating the processes governing the final plasma FA profile.

FA distribution in plasma lipids

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The distribution of individual FA in plasma lipid classes (PL, TG, CE) in humans varies appreciably even among FA of the same class or metabolic series, as it is for instance shown in Table 1. Of the total circulating AA, the greatest proportion is associated with PL, followed by CE, and minimal amounts are found in TG, whereas LA is mostly associated with CE, followed by PL and TG. Marked differences are found also in the distribution of DHA (mainly associated with PL) and EPA (largely associated with CE). These differences may affect the relative incorporation and exchanges of individual FA with cell lipids.

Table 1. Concentrations and % levels of individual FA in plasma lipid classes in 20 women.

FA	µg/ml	% distribution PL	TG	CE
18:2	640±125	34±6	10±5	56±9
20:4	148±39	67±6	4±2	29±7
20:5	9.6±3.9	57±12	11±10	31±12
22:6	28.8±9.8	85±5	7±7	8±3

FA correlations

Evaluation of the product/precursors relationships within the n-6 and n-3 FA series in Tanzanian populations on low fat diets (7-12 en%), strict vegetarians (VD) and fish eaters (FD) ingesting relatively high amounts of AA and DHA (typical in tropical fish) revealed the following : in VD only good correlations are present in the n-6 pathway (from LA to AA and especially between DHGLA and AA), and between ALA and EPA, in the n-3 series. In FD, significant correlations are found only between LA and DHGLA in the n-6 series, and between EPA and DHA in the n-3. These findings will be discussed in the context of the contributions of the exogenous supply of preformed LCP (FD and omnivores, in general) vs that of the endogenous biosynthesis exclusively The correlations in omnivorous Italian populations (>30 en % fat), are somewhat intermediate between those in the two Tanzanian populations.

Evaluation of the correlations between SAT, MUFA and PUFA in the three populations at study, revealed that : a. there is no correlation between SAT and MUFA, weak but significant negative correlations between PUFA and SAT (SAT vs PUFA : $y=42.6-0.24x$, $r=0.535$, $p < 0.001$ in Italians; $y = 48-0.38x$, $r=0.62$, $p<0.001$ in VD Tanzanians; $y= 46.2-0.29x$, $r=0.47$, $p<0.001$ in FD Tanzanians), very strong negative correlations between MUFA and PUFA (MUFA vs PUFA : $y=58.4-0.75x$,

r=0.89 in Italians; y=53.8 - 0.71x, r=0.80 in VD and y=51.9 - 0.62x, r=0.79 in FD). These data obtained in populations on diets with quantitatively and qualitatively very different fat contents, fit and are in agreement with the hypothesis that PUFA and MUFA compete for esterification, whereas this does not occur between SAT and MUFA. Additional relationships which will be discussed concern those between n-6 and n-3 levels. These, in plasma, at difference with the situation in cellular lipids, do not appear to be reciprocally modulated.

In a controlled clinical study with subjects on isocaloric diets (25 en% fat) with defined FA proportions (prudent diet, olive oil based diet and corn oil based diet) we have evaluated the relationships between dietary SAT, MUFA and PUFA as en% and the same FA classes as % of plasma FA. It appeared that differences in dietary SAT between 5 to 9.6 en% result in no difference in plasma SAT (% of total FA), whereas differences in dietary MUFA and PUFA result in proportional changes in the corresponding plasma FA.

Additional evaluations on the relationships between levels of FA classes as well as of individual FA, on one side, and plasma cholesterol and TG, on the other, reveal that correlations are present only with TG.

In conclusion, the observation of selected correlations among plasma FA, based on detailed analytical data, facilitates the interpretation of the dietary and metabolic relationships between FA and plasma lipids.

Nutritional and Metabolic Interrelationships Between Omega-3 Fatty Acids and Trans Fatty Acids

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Much attention on the health concerns of current intakes of trans fatty acids (TFA) via fast and processed foods has focused upon the potential for dietary TFA to significantly increase the LDL-cholesterol level while lowering HDL-cholesterol as well as increasing triglyceride and lipoprotein(a) levels in some studies. Epidemiological studies have indicated that TFA represent a major dietary risk factor for cardiovascular disease (CVD) in the North American population. A lesser focus has been placed on the potential for dietary TFA to interfere with the convertibility of linoleic acid and alpha-linolenic acid (α-LNA) to their longer-chain metabolic products. There is also evidence that TFAs may impair early growth in humans by impeding desaturation/elongation reactions. Recent data from Health Canada (Ratnayake and Chen) has indicated that the mean TFA intake (as trans 18:1) in Canadian adults represents 3.7% of total daily energy which is above recent estimated intakes of TFA for the US population (Allison et al, J. Am. Diet. Assoc., 1999). Young males in Canada (age 18-34 years) have a mean trans-18:1 intake of 12.5 g/day with intakes as high as 39 g/person/day. One of the richest sources of TFA in the Canadian food supply is breast milk from mothers who show a mean content of total TFA representing 7.2% of total fatty acids (and up to 17.2%). Furthermore, the total TFA: α-LNA ratio in Canadian breast milk is 6.2 to 1. These high ratios reflect the very high ratio of TFA:n-3 fatty acids in the diet of pregnant and lactating women. We have analyzed a wide variety of processed and fast foods in Canada (showing very high ratios of TFA:n-3 PUFA) as well as a wide variety of baby foods (cereals and biscuits) which, in many cases, show extremely high ratios

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of TFA:n-3 fatty acids. Foods containing hydrogenated vegetable oils which greatly compromise the n-3 fatty acids intake while enhancing the TFA consumption, as well as the potential for TFA to interfere with the convertibility of α-LNA to docosahexaenoic acid (DHA), likely accounts for the lower DHA status in humans consuming higher intakes of processed and fast foods containing TFA. Mandatory food labeling in North America for TFA and omega-3 fatty acids is needed to allow consumers to reduce the consumption of the former while increasing the latter. Such regulatory changes can be expected to enhance the physiological DHA status and related human health parameters beginning at conception.

Choice of n-3, Monounsaturated and Trans Fatty Acid-Enriched Oils

for the Prevention of Excessive Linoleic Acid Syndrome

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Excessive linoleic acid intake and relative n-3 deficiency syndrome

Animal experiments and epidemiological studies have revealed that excessive intake of linoleic acid (LA, n-6) is a major risk factor for cancers of western type, allergic hyper-reactivity, coronary heart disease (CHD) and cerebrovascular disease (CVD) (1). Although epidemiological studies performed in the USA failed to reveal a positive correlation between LA intake and breast cancer mortality, this is probably because the proximate marker for breast cancer is the proportion of n-6 eicosanoid precursors in phospholipids, which is saturated both in the high and low LA intake groups in the USA. Empirical equations presented by Lands indicate that both increasing the intake of n-3 fatty acids and decreasing that of n-6 fatty acids are necessary for effectively decreasing the n-6 eicosanoid precursors in phospholipids and thereby decreasing cancer mortality. On the other hand, high n-6/n-3 ratio but not hypercholesterolemia has been proved clinically to be a major risk factor for thrombotic diseases. Over-production of inflammatory lipid mediators of n-6 series has been shown to be a major cause for the rapid increase in allergic hyper-reactive patients in Japan.

Presidentís Summary 1997 from the Japan Society for Lipid Nutrition

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After discussion through several annual meetings of the Japan Society for Lipid Nutrition, Presidents Summary 1997 was published (in Japanese) as a review article (J. Lipid Nutr. 6:5-42, 1997), in which 20% as total fat energy was recommended for those with moderate physical activity. For healthy populations, saturated plus monounsaturated : n-6 : n-3 = 2.5 : ≤ 0.8 : ≥ 0.2 (n-6/n-3 ≤ 4) was recommended. For the primary and secondary prevention of those diseases described above, an n-6/n-3 ratio of 2 was recommended. The latter value was based on: 1) even the n-6/n-3 ratio of Danes was 3 in a well known epidemiology of Greenland natives; 2) the ratio of current Japanese is 4 but the incidence of cancers of western type has been increasing rapidly, and the ratio of 4 or above cannot be recommended; 3) animal experiments have shown the effectiveness of decreasing n-6/n-3 ratio to below 2 for the suppression of carcinogenesis and metastasis; and 4) the safety of n-6/n-3 ratio of 1 has been established in animal experiments and in a retrospective study on hunters and gatherersí foods.

In order to meet the recommendations described above, vegetable oils with n-6/n-3 ratios of 2 or below and those with very low n-6 fatty acid contents (e.g., high-oleic type) are useful. However, there was another criterion to be considered; the presence of minor components which affect animal physiology seriously.

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Survival time-shortening and renal injury induced by some vegetable oils and partially hydrogenated oils in SHRSP rats

Using soybean oil as a control, some oils were found to prolong the mean survival time of SHRSP rats by ca 10% (e.g., DHA-rich fish oil, perilla seed oil, flaxseed oil) while some others shortened it dose-dependently by ca 40% (double-low rapeseed oil, evening primose oil, high-oleate safflower oil, high-oleate sunflower oil, olive oil and partially hydrogenated rapeseed and soybean oil). When the rapeseed oil was lipase-treated, the resulting free fatty acid fraction was almost free of such activity, indicating that the survival-time shortening activity is due to minor components other than fatty acids in these oils. Free fatty acid fraction from partially-hydrogenated soybean oil exhibited a survival time between those of the original oil and soybean oil. It should be emphasized that lard, sesame oil and high-linoleate safflower oil were relatively safe for the SHRSP rats.

Those oils with survival-time shortening activity were found to cause renal injury; lesions in blood vessels, accelerated proteinuria, decreased platelet count and elevated gene expression for TGFβ, fibronectin and renin.

Choice of n-3, monounsaturated and trans fatty acid-enriched oils

In order to decrease the n-6/n-3 ratio of our current foods to 2 or below, the intake of high-α-linolenate oils such as perilla seed oil and flaxseed oil as well as seafood and vegetables should be increased. High-linoleate oils are inappropriate for human use as foods. For deep-frying and preservation purpose, high-oleate vegetable oils are useful but all the high-oleate vegetable oils and hydrogenated vegetable oils we have examined so far exhibited the survival time-shortening activity,

and I cannot recommend people to have these oils in large quantities. Instead, lard was safe for this animal model, and could be used in quantities not to induce obesity; animal fats as well as a high-LA vegetable oil intake caused insulin resistance in a NIDDM model of rats.

Reference

Okuyama, H., Kobayashi, T., and Watanabe, S. (1997) Dietary fatty acids ñ The n-6/n-3 balance and chronic, elderly diseases. Excess linoleic acid and relative n-3 deficiency syndrome seen in Japan. Prog. Lipid Res. 35:409-457.

Friday, April 9, 1999

Session V. Dietary Recommendations and Omega-6:Omega-3 Ratio

Intakes of Dietary Fatty Acid in the United States: Results from the USDA's 1994-1996 Continuing Survey of Food Intakes by Individuals

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The USDA has been conducting biennial food intake surveys for many years. In the more recent surveys individual fatty acid intakes were estimated from food composition data accumulated and published in the USDA Handbook 8, "The Composition of Foods." In the compilation from the combined 1994 and 1996 surveys, the USDA has published data on 19

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individual fatty acids in the US diet. Included are all the saturated fatty acids from C-4 to C-18, the monounsaturated fatty acids 16:1, 18:1, 20:1, and 22:1 and the polyunsaturated fatty acids 18:2, 18:3, 18:4, 20:4, 20:5, 22:5, and 22:6. The data is broken down by sex and age from less than 1 year to more than 70 and by percent of calories or grams per day. Although there are some age dependent trends in the consumption data, the majority of the population does not show significant differences between five and sixty years of age, and only slight differences due to sex. In the USA 11 percent of calories are consumed from saturated fat, 13 percent as monounsaturated fat, and 6 percent as polyunsaturated fat. Oleic acid is the preponderant monounsaturated fatty acid, and linoleic acid is the major polyunsaturated fatty acid. The USDA data for monounsaturated fatty acid presumably include trans isomers of monounsaturated fatty acids and are grouped together with 18:1 fatty acids. Current calculations using the best available estimated of trans fatty acid C-18 isomers in the foods consumed by the US population suggest that the actual consumption of trans configuration fatty acids is 3 percent and cis-monounsaturated fatty acids is about 10 percent. The saturated fatty acid category exhibits a broader distribution of fatty acids consumed than that observed for the unsaturated fatty acids; 12:0, 14:0, 16:0 and 18:0 all contribute significantly to the fat calorie intake in the US population. Palmitic acid accounts for 20 percent of fat calories and stearic acids about 9 percent. Of particular interest to this workshop is the intake of long-chain polyunsaturated fatty acids, especially those with twenty or more carbon atoms in the fatty acid chain. Unfortunately, the USDA data contain relatively little information on this topic. Due to the nature of survey information and the sparsity on information regarding long-chain polyunsaturated fatty acids in the food composition data from which the tables are prepared, no detailed view of the intake of omega-3 fatty acids can be made. The data do show that the US population consumes approximately 10 times the amount of omega-6 fatty acids as omega-3 (the ratio is 0.11 n3/n6),

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but it is probable that the USDA data underestimated the omega-3 intake. In terms of grams per day the mean intake of linoleic acid plus arachidonic acid is 13.0 while the intake of α-linolenic acid plus docosahexaenoic acid is 1.5. The ratio of docosahexaenoic acid to arachidonic is, however, 1 (0.1 to 0.1 grams per day). As it is not possible to demonstrate an omega-3 fatty acid deficiency in the US population, the intake of 2 grams per day of omega-3 fatty acids must be at least the required daily intake when the intake of omega-6 is 20 grams per day. Of course, 13 grams per day of omega-6 fatty acids are likely to be considerably more than the required daily intake. Evidence from animals, and limited human data, suggests that the required daily intake is likely to be less than 5 grams per day (2 percent of calories). Whether the required daily intake of omega-3 fatty acids would be less if less omega-6 fatty acids were being consumed is unknown. If the total fat intake is reduced, it may be necessary to increase the intake of omega-3 fatty acids to avoid omega-3 fatty acid deficiency. Neither the USDA nor the federal Government have a recommendation for the DRI of polyunsaturated fatty acids presently. It is unlikely that a single amount could be recommended for all age ranges. The requirement for omega-3 fatty acids will probably be age dependent. Whether there is an absolute requirement for polyunsaturated fatty acids with twenty or more carbons in the chain remains to be determined.

World Health Organization/Pan American Health Organization

(Status of EFA Worldwide)

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The three main nutritional problems in the world are protein-energy malnutrition (PEM), micronutrient deficiencies (iron, vitamin A, iodine and folic acid) and overweight/obesity.

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Stunting is the most common expression of PEM, but other forms of PEM are equally frequent among children below two years of age and child-bearing women.

Iron deficiency is the most widespread nutritional problem. Vitamin A and iodine deficiency showed a declining trend during the past years as a result of supplementation and fortification strategies, carried-out in countries.

Obesity is increasing worldwide at an alarming rate in both developed and developing countries. This situation is associated with rapid changes in dietary patterns and lifestyles.

Data from several countries show relatively high prevalence of obesity, particularly in women from poor urban areas. Furthermore, a sharp increase in morbidity and mortality rates due to nutrition-related non-communicable diseases has been reported.

One of the most important factors underlying this scenario among low socio-economic groups is the increase in energy intake associated with higher fat and refined carbohydrate consumption accompanied by a low iron, zinc, and folic acid intake.

Very little information exists on quality and composition of fats by low social-economic groups in the majority of countries.

Information in this area is of utmost importance to guide the selection and consumption of healthy diets as part of the Health Promotion strategy of PAHO/WHO.

n-3 Fatty Acids: Food Supply, Food Composition and Food Consumption Data

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Food supply data, food composition data, and food consumption data provide a fundamental basis for assessing the health and nutritional adequacy of individuals and populations. FAO has the UN mandate for these activities, and regularly produces Food Balance Sheets (FBS), which provide food supply data, including selected nutrient values. This paper will highlight estimates of available n-3 fatty acid containing foods from around the world. Most commonly, the nutrient data from FBS are expressed only in terms of energy, protein and fat. These international "default" nutrient values are being revised and the list of nutrients is now being expanded to include several micronutrients. In 1998, the cereals group was completed, and in 1999 the fish group will be revised. Under discussion is the possibility of including fatty acids and/or n-3 fatty acids as a special nutrient category for the fish group. Food composition activities in FAO come under the auspices of the FAO/UNU INFOODS project. We are providing assistance in all technical aspects of food composition. More and more frequently, fatty acids are included among the nutrients shortlisted by countries for inclusion in their national and regional food composition databases and tables. The inclusion of fatty acids is based on requests from the countriesí users and potential users of food composition data, and

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the countriesí diet-related morbidity and mortality statistics. Commonly used laboratory instruments (gas chromatographs), well-defined analytical methodologies, and the availability of primary and secondary reference materials and standards, make analysis of n-3 fatty acids a routine activity for many laboratories. Data on n-3 fatty acids are now being generated, compiled and disseminated in many countries, including many developing countries. Sources and quantities of n-3 fatty acids will be presented. Food consumption data are routinely used, along with food supply data and food composition data, to establish food security at household, district and national levels. FAO prepares Nutrition Country profiles, which to date have not included assessment of n-3 fatty acids. However, now that acceptable quantities of high quality n-3 fatty acid data are becoming available from food composition laboratories, n-3 values can be incorporated into supply data, and food consumption studies will in the next few years be capable of reporting the n-3 fatty acid consumption in the assessments of food security.

BASFís Approach to Commercialization of Long Chain Omega-3 Fatty Acids

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The goal to deliver omega-3 fish oils without adverse taste, odor and to prevent oxidative degradation has been a formulation objective. Utilizing spray-cooling technology for microencapsulating highly refined, deodorized and stabilized fish oils has proven to be successful in regard to producing powdered products that can be used to formulate fish oils into most all conventional food forms. Formulated food products, such as pastas, cereals, and even beverages, can be formulated with microencapsulated fish oils at levels of about 100 mg LCPUFAs per 100 gram product without detection of their inclusion.

The powdered microencapsulated product has been evaluated in clinical investigations to confirm its equivalency to the bioavailability of oils. Studies are being conducted to establish the productís use in formulated food form to deliver meaningful amounts of omega-3 fatty acids to pregnant women and ultimately to their breast fed infants.

BASF is enhancing its activities to educate and promote the incorporation of long chain omega-3 fatty acids to the food industry in a variety of ways: participating in the scientific community by funding studies and providing test materials, developing educational materials for health care providers as well as retailers and the consumer, promoting the use of omega-3ís through advertisements and public relation activities, and developing a trademark to help draw attention to the incorporation of a unique food ingredient.

BASF will continue to support trade and professional associations working towards the establishment of Dietary Reference Intakes and health claim allowances.

Essential Fatty Acids and the Products of the Groupe Danone for Human Nutrition

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Recent evaluations on the intake of fat in France show it to be in the range of 38 % of total calories : 50% of that fat is hidden in raw materials (27 % in meat and fish, 17 % in dairy products, 6 % in fruit and vegetables). The other 50% of the lipids consumed are added directly to the recipes; 30 % by the consumers themselves (butter, margarine, oil), 20 % by the food industries (meal, sausage, biscuitsÖ). Nevertheless, in recent years the intake of fat appears to be on the decline.

In an ongoing prospective study on 15,000 French people, it has been found that saturated fatty acids supply 17 % of calories, mono-unsaturated 14 % and polyunsaturated 6 % :

g/day	Men	Women
Total lipids	100.4	78.6
MUFA g/day	37.8	29.4
PUFA g/day	16.6	12.7
SFA g/ day	44.8	35.6

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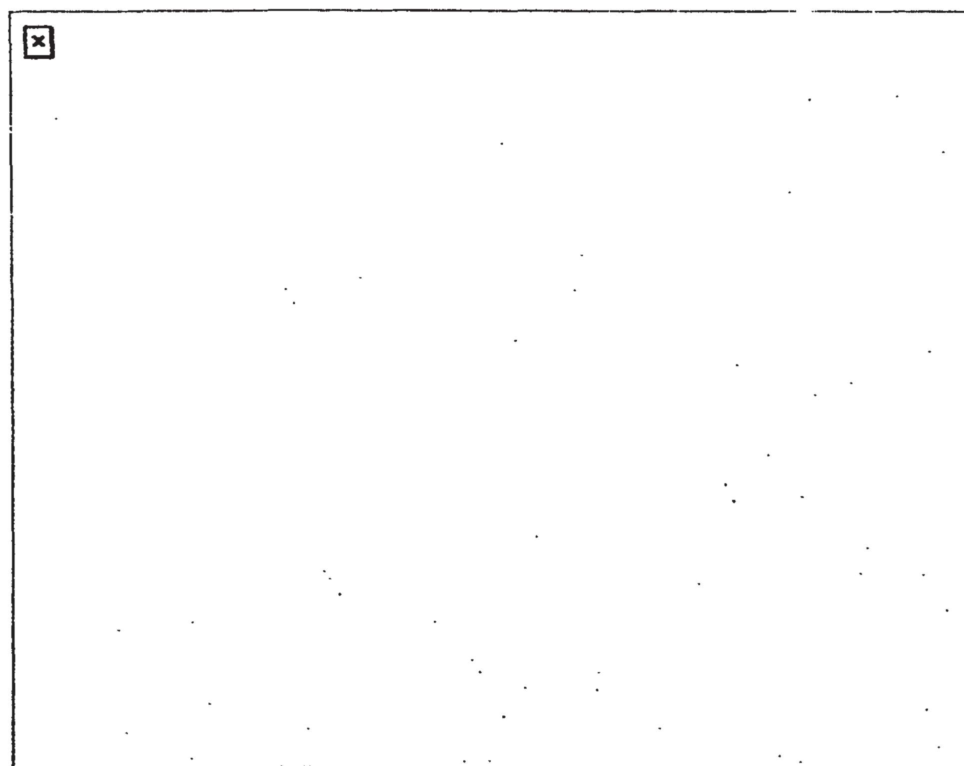
The ratio n-6/n-3 fatty acids is probably in the range of 15-20.

Over the last 30 years, recommendations have been changing in relation to the type of fatty acid to be included in the human diet and the total amount of fat suggested. The Danone group has been closely following this trend and has tried to adapt its products to the recent recommendations.



Trend in recommendations in fatty acids ratio : Danone approach

The present conclusion in France is that the population consumes too much saturated fat as well as too much n-6 polyunsaturated fatty acids.

Thus, the food industry has to change the fatty acid composition of its products to readjust the intake of fatty acids in the French population. Instead of using butter, tallow or different oils, canola can be utilized. Canola oil is a typical example of a fat containing a small amount of saturated fatty acids, and supplying both n-6 and n-3 polyunsaturated fatty acids in a proper ratio to counterbalance the fatty acid intake from meat, dairy products and other sources.

Thus, it seems fundamental that the experts in nutrition express clear recommendations in the field of fats and fatty acids, in relation to public health, since we, the food industry, will follow their recommendations.

Advantages and Disadvantages of the Use of Flax Seed as a Source of Omega-3

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Flax seed is presently being used worldwide as a source of Omega-3 in human and companion animalsí diets. History of the use of flax seed as food for humans goes back 2,000 years. Flax seed has several distinct advantages and disadvantages as a source of Omega-3.

Disadvantages of flax seed include such factors as:

1. Presence of "Anti B-6" factor.

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2. Presence of Cyanogenic Diglycosides.

3. Unstable after being ground.

4. Contains only short chain Omega-3.

Advantages of flax seed include such factors as:

1. High concentration of alpha-linolenic acid.

2. Presence of powerful anti-oxidants in some varieties.

3. Presence of high levels of soluble and insoluble fiber.

4. Presence of high levels of lignans that have anti-estrogenic properties.

5. FDA states, "no objection" as a food.

6. Desirable flavor in most foods.

Omega-3 LC-PUFA - from a Health Concept to Foods in the Shelves

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Incorporating long-chain polyunsaturated fatty acids (LC-PUFA) into the diet, continues to be a topic of interest among food manufacturers. Nutritionists believe that addition of omega-3 LC-PUFA - eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) - to the diet would result in better nutrition and assist in chronic disease prevention. General scientific opinion appears to be that as little as 200-1000 mg of EPA/DHA may achieve this dietary goal.

The refining technology for marine oils has advanced to the degree that, with due care, careful handling and proper precautions, it is now possible to produce a variety of foodstuffs fortified with omega-3 LC-PUFA that taste as good as similar, unfortified products.

At the forefront of developments are infant formula and baby follow-on food in Europe and the Far East. In addition, breads, margarines (or other low-fat spreads), UHT milks, yogurts, fruit juices and beverages have started to enter the mainstream in Europe. Niche products such as soups, salad dressings, mayonnaise, ice tea drinks, cakes, biscuits and the restoration of omega-3 LC-PUFA to canned seafood and tuna are being launched.

Despite of this growing list, many food manufacturers are still reluctant to develop products fortified with omega-3 LC-PUFA due to the following non-technical barriers:

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Recommendations: There are no officially recognized intake recommendations for omega- 3 LC- PUFA. The food manufacturer has no standard of reference for the nutritional value or dietary fortification levels of omega-3 LC-PUFA.

Claims: No product label or health claims are permitted by the FDA and other regulatory authorities, which makes it extremely difficult to market a food with omega-3 LC-PUFA.

Safety: There is an unwarranted fear of allergenicity and the possible effects of omega-3 LC-PUFA on bleeding and insulin resistance. There are numerous reports that such adverse reactions do not occur even at the maximum dosages of omega-3 LC-PUFA which are considered to be health beneficial (1-2g).

Awareness: The awareness about the health benefits of omega-3 LC-PUFA is generally poor among consumers. The closing of this knowledge gap is made difficult by the bewildering number of and sometimes complicated names for the family of omega-3 LC- PUFA and its members e.g. PUFA, HUFA, n- 3 LCP, omega- 3 LC- PUFA, EPA, DHA, eicosapentaenoic acid, docosahexaenoic acid.

To make omega-3 LC-PUFA a standard food ingredient it is imperative that food industry suppliers, food manufacturers and professional organizations (such as ISSFAL) work hand in hand to remove these obstacles by providing authorities, health professionals and the public with truthful, scientifically valid information about the health benefits of omega-3 LC-PUFA.

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Infant Formulas with no DHA or ARA. Are They Causing Harm?

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Over the past twenty years there have been a large number of retrospective studies comparing the neurological outcomes of breast-fed and formula-fed infants. A recent meta-analysis of the most relevant of these studies has indicated that there is a consistent 3-4 IQ point advantage to the breast-fed infants even after the contributions of all other confounding factors had been removed. Breast-fed babies, however, are getting many nutrients from the breast milk in addition to docosahexaenoic acid (DHA) and arachidonic acid (ARA) and many have argued that the contribution of DHA and ARA is

inconclusive. One observation, however, is very clear and consistent. Infants who are provided standard infant formula have significant deviations in their blood and brain biochemistry relative to the breast-fed babies. Full term infants fed unsupplemented formulas have a circulating DHA status (as indicated by red blood cell or plasma phospholipid DHA levels) of less than one-half that of the breast-fed infant. Furthermore, the brain DHA levels of formula-fed infants are about one-third lower than those of

breast-fed infants.

Since some groups have also argued that such changes in the blood and brain biochemistry in the formula-fed infant is irrelevant, it has been critically important to more fully understand the function of DHA in the tissues of the body. Recent studies have revealed that DHA has many critical functions in the normal development and metabolism of neuronal cells. These include, but are not limited to, the following: 1) the control of normal migration of neurones from the surface of the ventricles of the brain to the cortical plate during development; 2) the control of the normal resting potential of the neurone by regulation of sodium and calcium channels; 3) the regulation of the density of certain membrane proteins such as rhodopsin in the retina and, possibly, 4) the regulation of levels of certain neurotransmitters such as serotonin. With such key roles in normal neuronal development and function, it is quite plausible that abnormally low levels of this primary nutrient during the development of the brain may be one cause of the long term neurological detriments observed in formula-fed infants relative to breast-fed infants.

The final proof of the importance of DHA in early infant nutrition, however, comes not from demonstrating that the long term neurological outcome of formula-fed infants is poorer than breast-fed infants, or that this poor outcome is correlated with a DHA deficiency early in life, but from interventional studies which demonstrate that when the DHA deficiency is removed, the neurological outcomes revert to normal. There have been at least 24 well-controlled studies involving over 2,000 infants in the last 15 years (12 studies with term infants and 12 studies with pre-term infants) which have compared outcomes of standard formula-fed infants with DHA-supplemented formula-fed infants. In every study the DHA status of the infants was returned to normal (as defined by the DHA status of the breast-fed infants) when the formulas were supplemented with DHA. In all of these studies, except where fish oil was used as a source of DHA, the ARA levels were also normalized because of the use of supplemental ARA in the formulas. In several studies, precursors such as gamma-linolenic acid (GLA) or alpha-linolenic acid (ALA) were added to the formulas in an attempt to elevate ARA or DHA levels respectively. Even when added in significant excesses over what is found in breast milk however, these precursors did not elevate the DHA and ARA levels to those of the breast-fed infant. That is, the precursors do not adequately substitute for the preformed DHA and ARA provided in motherís milk. Of all the trials completed with DHA/ARA supplementation, single cell oils (SCOís) were used with the largest numbers of babies (45% with SCOís, 35% with egg yolk; and 20% with various fish oils).

Of the 24 DHA/ARA supplementation studies mentioned above, only 12 looked for functional outcomes differences (i.e., visual, neurological, or developmental assessments). Seven of those 12 studies reported statistically significant deficits in standard formula-fed babies compared to breast-fed babies (the gold standard). In all 7 cases, those deficits were normalized with the DHA/ARA supplementation. Of the remaining 5 studies, no statistically significant differences could be found between formula-fed and breast-fed babies using the test metrics employed in those studies and, therefore, no effect of DHA/ARA supplementation was observed.

The totality of these observations provide strong evidence that DHA is a critical nutritional requirement for the newborn infant and that an early deficiency of DHA could lead to long term neurological deficiencies. Given our present state of understanding, it is quite possible that the lack of availability of DHA and ARA-supplemented infant formulas in the United States and Canada today may be putting formula-fed newborn babies at risk. Since the only way that newborn babies in the United States and Canada can get DHA and ARA today is from their motherís milk, we must use our best efforts to encourage new mothers to nurse their babies for as long as possible to avoid potential long term neurological deficits to the child.

Clinical Safety Studies of LCPUFA Supplementation of

Premature and Term Infant Formulas

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Introduction: Many studies support a need for long chain polyunsaturated fatty acids (LCPUFA), and particularly docosahexaenoic acid (DHA, 22:6n-3), for optimal retinal and neural development in early infancy. Human milk contains LCPUFA, including DHA and arachidonic acid (ARA, 20:4n-6), but U.S. infant formulas do not. We have now completed two of the largest clinical trials of LCPUFA supplementation, one with very low birth weight infants and a second with healthy full term infants.

Premature Infant Study: DHA supplementation has been shown to enhance visual development of preterm infants, but some studies found decreased growth when DHA was provided without ARA. **Objectives:** (1) To establish the safety of feeding DHA and ARA from single cell oils to preterm infants and (2) to determine effects on visual acuity. **Design:** In a double-blind, controlled, multi-center trial, 194 preterm infants were randomized to preterm formulas differing only in fatty acid content: no DHA or ARA (control), 0.15 % (of energy) DHA, or 0.14 % DHA + 0.27 % ARA. Preterm formulas were fed for at least 28 days; all preterm infants then received unsupplemented term formula. Ninety breast-fed term infants were enrolled as a reference group. **Results:** Growth suppression was not seen in the DHA or DHA+ARA groups; in fact, post-hoc analyses indicated that weight gain of DHA+ARA infants was significantly enhanced compared to control. Weight of DHA+ARA infants was not different from breast-fed term infants at 48 and 57 wk postmenstrual age (PMA), but weight of control and DHA infants remained significantly less than breast-fed term infants through 57 wk PMA. There were no significant differences between preterm groups in incidence of serious adverse events, NEC/suspected NEC, or sepsis/suspected sepsis. Visual acuity determined by Teller Acuity Cards (TAC) at 48 and 57 wk PMA did not differ among preterm groups. **Conclusions:** Single cell oils are safe for use in preterm infant formulas to provide DHA and ARA at human milk levels. Providing DHA plus ARA enhances catch-up growth of premature infants; however, supplementation for 28 days did not affect TAC acuity 3 and 5 months later.

Term Infant Study: Studies of LCPUFA supplementation of formula-fed term infants have shown equivocal effects on visual and cognitive development, but several recent studies with typical human

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milk levels of DHA have found beneficial effects. Because term formulas may be fed for a full year, the safety of LCPUFA supplementation over this time period must be established. **Objectives:** (1) To establish the safety of feeding DHA from single cell and fish oil sources, each in combination with ARA from single cell oil, to term infants to a year of age and (2) to evaluate effects of supplemented formula on visual acuity and mental and psychomotor development. **Design:** In a double-blind, multi-center trial, 383 term infants were randomized to formulas differing in fatty acid content: no LCPUFA (control), 0.15% (of energy) DHA and 0.3% ARA from single cell oils, or 0.15% DHA from fish oil and 0.3% ARA from single cell oil. **Results:** Weight gain from day 14 to days 60 or 120 was not significantly less in supplemented groups compared with the control. Furthermore, post-hoc analyses indicated that supplemented infants had larger growth rates than control infants from 14 to 60 and 120 days. No differences were observed in mean weight, length or head circumference at 180, 270, or 365 days; in formula acceptance and tolerance; or in incidence of serious adverse events. No differences were observed in visual acuity (TAC) at 120, 180, and 365 days or in Bayley MDI and PDI scores at 365 days, although Bayley scores were somewhat higher in supplemented groups than in the control. **Conclusions:** DHA from single cell oils and ARA from single cell oil are safe for use in term infant formulas when fed at human milk levels for a full year. Supplementation with DHA and ARA increased early growth of term infants, similar to our findings with preterm infants, but did not significantly affect TAC acuity or mental or psychomotor development.

Overall Conclusions: Our large clinical trials, along with numerous other clinical and toxicology studies, demonstrate the safety of adding typical human milk levels of DHA and ARA to both premature and term infant formulas over the time periods these formulas are typically fed. While our trials did not find significant benefits of LCPUFA supplementation for visual and cognitive development, we did find increased growth in both premature and term infants supplemented with DHA plus ARA. This increased growth may be particularly important with regards to enhancing catch-up growth of infants born prematurely.

Omega-3 Long Chain PUFA ñ Closing the Nutritional Gap

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Significant research shows that the populations of many industrialized nations, including the U.S., consume significantly lower levels of omega-3 long chain PUFA than science shows is required for maintaining good health. There needs to be a concerted effort by industry, government and the scientific community to ensure that this nutritional gap is eliminated.

Trends - The Time to Act is Now

There is significant momentum and steam building that highlights the need for a cooperative effort in ensuring that the populations benefit from the improved science and manufacturing capabilities now in place.

Some of the important trends taking shape include:

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? The improved manufacturing capabilities that permit the fortification of good tasting, stable food products as seen in many parts of the world;

? The ability to manufacture highly concentrated oils that can be delivered as adjunctive therapies;

? The increasing awareness (54% - Applied Biometrics, October 1998) of consumers with respect to the health benefits of omega-3 that now needs to be converted into usage;

? Improved collaboration between industry and science;

? Improved science showing the benefits of increased consumption of omega-3 LC-PUFA.

Steps to Success

In order to ensure that consumers benefit from the science, it is going to be essential that officially recognized intake levels are set for omega-3 LC-PUFA. Omega-3 LC-PUFA will not gain mass-market acceptance or incorporation into standard food channels until the manufacturers have an officially recognized reference point and/or the ability to make an approved health claim.

Key steps to success:

? Establish officially recognized intake recommendations for omega-3 LC-PUFA that manufacturers can reference on the label;

? An FDA-approved health claim for omega-3 LC-PUFA with reference to cardiovascular health and triglyceride lowering;

? A better understanding of the correct omega-6 to omega-3 ratios and the upper and lower limits based on age and health status;

? The standardization of analytical methods to ensure consumers and industry are able to make true product comparisons against the science;

? Quality standards enforced to ensure that consumers are not exposed to substandard product with contaminants or oxidative problems.

To make omega-3 LC-PUFA a standard food ingredient, the time to act is now. We need to form partnerships between industry suppliers, food manufacturers, professional organizations and the government. The goal is to utilize the present market conditions in an effort to ensure that consumers are given the best opportunity at better nutrition through the proper balance and total consumption of omega-3 LC-PUFA.

Safety of Omega-3 Products Based on Fish Oil as Starting Material

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Pronova is the largest producer of omega-3 products in the world today with products ranging from pharmaceuticals via medium concentrated food supplements to refined crude fish oil.

Convincing monitoring of safety is only possible in controlled clinical studies, preferably by so-called Good Clinical Practice studies. The present database of patients in controlled studies on active treatment comprises more than 9000 individuals mainly in long-term studies of more than one year; 60% more than 3.5 years. This study population consists of patients with chronic diseases related to the cardiovascular and renal system but also diabetics with age ranging from early adolescence up to 70 years and more.

Today we have no report of serious adverse effects, whatsoever. Even if bleeding time has been prolonged with omega-3 products, there are no reports of serious bleeding events even in patients on concomitant medication with Aspirin or Warfarin. Adverse effects are seen in 10-20% of the patients in studies mainly originating from the GI tract. Eructation of fishy taste is the most common finding. Interestingly, the frequency of eructation is the same in the placebo group receiving corn oil as in the active treatment group indicating that eructation is a function of ingesting oil in general. Studies including diabetics with a total number of approximately 1500 patients have not shown derangement of diabetic control. Patients with chronic renal disease, renal failure and even transplanted patients on chronic cyclosporin medication have not shown any systemic adverse effects but rather an improvement of renal function. In studies on pregnant women there have been no bleeding complications and the amount of bleeding during labour has not been significantly different from controls.

The regulatory authorities in countries like the US and several EU countries have examined the safety file of the pharmaceutical, Omacor, and there have been no major objections. Omacor is a registered pharmaceutical in several EU countries and an application for an NDA in the US is planned for later this year. At the recent American College of Cardiology meeting in New Orleans, the results of GISSI Prevention were presented. This is a study including 11,324 post-MI patients comparing 1g of Omacor, vitamin E and the combination with a control group. All patients were optimally treated with aspirin, beta-blockers, statins, etc. The Omacor group but not vitamin E showed a 20% reduction of mortality, and treatment was very well tolerated. Conducted by the prestigious Mario Negri Institute of Milan, Italy, this study is the most important documentation of efficacy and safety for any omega-3 product in the world today.

An interesting adverse report from one patient on omega-3 treatment in Houston, USA was an "urge to swim". We take this more as a joke but we would like to use this metaphor claiming that products using fish oil as starting material, and therefore containing both EPA and DHA, are the state-of-art today and based on a natural dietary principle and accepted by regulatory authorities as safe during long term use. Pure DHA products are expensive and the DHA content will readily be retro-converted to EPA in humans to meet metabolic needs. Mechanistic studies on separate effects of EPA or DHA will have to be conducted in *in vitro* systems but the results will have only minor impact on therapy traditions using omega-3 products introduced today.

In conclusion, Pronova, as the worldís largest producer of omega-3 products using fish oil as starting material, holds the largest database on safety as well as efficacy in patients and healthy individuals today. These products are regarded as safe when used either as pharmaceuticals, food supplements, or

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in fortification of food products.

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