AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Massachusetts on the following ☒ Patents or ☐ Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:09-cv-11946	11/13/2009	District of Massachusetts

PLAINTIFF	DEFENDANT
Neptune Technologies & Bioressources, Inc. & L'Universite de Sherbrooke	Aker BioMarine ASA, Jedwards International, Inc., & Virgin Antarctic LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	6,800,299	10/5/2004	L'Universite de Sherbrooke
2			
3			
4			
5			

In the above—entitled case, the following patent(s)/trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading		
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1			
2			
3			
4			
5			

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

NEPN Ex. 2044
Aker v. Neptune
IPR2014-00003

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,800,299 B1
APPLICATION NO. : 09/830146
DATED : October 5, 2004
INVENTOR(S) : Beaudoin et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page #75 INVENTORS, replace "Sherbrooke" with --Quebec--

FOREIGN PATENT DOCUMENTS, Line 8 delete (the second listing of) "JP 04057853 2/1992"

FOREIGN PATENT DOCUMENTS, Line 9 delete "JP 08198754 6/1996"

OTHER PUBLICATIONS, Line 8 insert --Chemical Abstracts no. 177859, vol. 98, no. 21 "Isolation of astaxanthin from crayfish or shrimp waste for use as a coloring agent in fish feed" March 23, 1983.--

OTHER PUBLICATIONS, Col. 2 line 1 replace "Château" with --Château--

OTHER PUBLICATIONS, Col. 2 line 6 replace "Mitake" with --Mitaka--

OTHER PUBLICATIONS, Col. 2 line 11 replace "conbinatorial" with --combinatorial--

OTHER PUBLICATIONS, Col. 2 line 15 replace "Alvarea" with --Alvarez--

OTHER PUBLICATIONS, Col. 2 line 15 replace "Arangea" with --Aranega--

OTHER PUBLICATIONS, Col. 2 line 28 replace "Granzow.." with --Granzow.--

OTHER PUBLICATIONS, Col. 2 line 31 replace "DiPrimo" with --DiPrimio--

OTHER PUBLICATIONS, Col. 2 line 34 replace "Curevo" with --Cuervo--

Column 5, line 40, make new paragraph at "Table 1"

Column 8, line 29, replace "with" with --to--

Column 9, line 15, replace "Heligren" with --Hellgren--

Column 11, line 6, replace "(M. norv gica)" with --(M. norvegica)--

Column 11, line 51, replace "(Macker l) with --(Mackerel)--

Column 12, line 4, replace "(Macker l)" with --(Mackerel)--.

UNITED STATES PATENT AND TRADEMARK OFFICE

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PATENT NO. : 6,800,299 B1
APPLICATION NO. : 09/830146
DATED : October 5, 2004
INVENTOR(S) : Beaudoin et al.

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Column 12, line 55, replace "4°." with --4° C.--

Column 13, line 27, insert --3-- before "chlor:MeOHd"

Column 13, line 44, insert --3-- before "chlor:MeOHd"

Column 13, line 63, "Wijs iodine index" should be underlined

Column 16, line 15, replace "0.1" with --0.2--

Column 16, line 38, replace "16:3 n-3 + I 17:1" with --16:3 n-4 + I 17:1--

Column 17, line 12, replace "Fraction Ib" with --Fraction Ilb--

Column 19, line 51, replace "nitration" with --filtration--

Column 20, line 22, replace "try" with --by--

Signed and Sealed this

Ninth Day of January, 2007

JON W. DUDAS
Director of the United States Patent and Trademark Office

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: BEAUDOIN et al.

Patent No. 6,800,299 Serial No: 09/830,146

Issued: October 5, 2004 Filed: July 25, 2001

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

TRANSMITTAL LETTER

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 11-15-06.

Reg. No. 40,764
Mark D. Passler

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Please find enclosed for filing the following:

X Request for Certificate of Correction, with form PTO/SB/44 (in duplicate)
X Check in the amount of $100.00
X Postcard

Please charge any underpayment or credit any overpayment to Deposit Account No. 50-0951. This transmittal letter is submitted in duplicate.

Respectfully submitted,

Dated: 11-15-06

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
222 Lakeview Avenue, 4th Floor
P.O. Box 3188
West Palm Beach, FL 33402-3188
Tel: 561-653-5000

Docket No. 789-47

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: BEAUDOIN et al.

Patent No. 6,800,299 Serial No: 09/830,146

Issued: October 5, 2004 Filed: July 25, 2001

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER RULE 1.323

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 11-15-06

Reg. No. 40,764
Mark D. Passler

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, on behalf of the Applicant, respectfully requests that a Certificate of Correction be issued on the above-identified patent.

The requested corrections are indicated, in appropriate form, on attached form PTO/SB/44, submitted in duplicate. The errors are as follows:

Page 1, INVENTORS, replace "Sherbrooke" with --Quebec--

Page 1, FOREIGN PATENT DOCUMENTS, delete (the second listing of) "JP 04057853 2/1992"

Page 1, FOREIGN PATENT DOCUMENTS, delete "JP 08198754 6/1996"

Page 1, OTHER PUBLICATIONS, insert --Chemical Abstracts no. 177859, vol. 98, no. 21 "Isolation of astaxanthin from crayfish or shrimp waste for use as a coloring agent in fish feed" March 23, 1983.--

Page 1, OTHER PUBLICATIONS, replace "Château" with --Château--

Page 1, OTHER PUBLICATIONS, replace "Mitake" with --Mitaka--

Page 1, OTHER PUBLICATIONS, replace "conbinatorial" with --combinatorial--

Page 1, OTHER PUBLICATIONS, replace "Alvarea" with --Alvarez--

Page 1, OTHER PUBLICATIONS, replace "Arangea" with --Aranega--

Page 1, OTHER PUBLICATIONS, replace "Granzow.." with --Granzow.--

Page 1, OTHER PUBLICATIONS, replace "DiPrimo" with --DiPrimio--

Page 1, OTHER PUBLICATIONS, replace "Curevo" with --Cuervo--

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Column 9, line 15, replace "Heligren" with --Hellgren--

Column 11, line 6, replace "(M. norv gica)" with --(M. norvegica)--

Column 11, line 51, replace "(Macker I)" with --(Mackerel)--

Column 12, line 4, replace "(Macker I)" with --(Mackerel)--

Column 12, line 55, replace "4°." with --4° C.--

Column 13, line 27, insert --3-- before chlor:MeOH[d]

Column 13, line 44, insert --3-- before chlor:MeOH[d]

Column 13, line 63, "Wijs iodine index" should be underlined

Column 16, line 15, replace "0.1" with --0.2--

Column 16, line 38, replace "16:3 n-3 + I 17:1" with --16:3 n-4 + I 17:1--

Column 17, line 12, replace "Fraction I^b" with --Fraction II^b--

Column 19, line 51, replace "nitration" with --filtration--

Column 20, line 22, replace "try" with --by--

It is respectfully requested that the Commissioner issue a Certificate of Correction in accordance with 37 C.F.R. §1.323. The appropriate fee in the amount of $100.00 is enclosed. Please charge any deficiencies or credit any overpayment of fees to Deposit Account No. 50-0951.

Respectfully submitted,

Dated: 11-15-06

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
222 Lakeview Avenue, 4th Floor
P.O. Box 3188
West Palm Beach, FL 33402-3188
Tel: 561-653-5000

Docket No. 789-47

PTO/SB/44 (04-05)
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(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,800,299

APPLICATION NO.: 09/830,146

ISSUE DATE : October 5, 2004

INVENTOR(S) : BEAUDOIN et al.

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Page 1, OTHER PUBLICATIONS, replace "DiPrimo" with --DiPrimio--

Page 1, OTHER PUBLICATIONS, replace "Curevo" with --Cuervo--

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Akerman Senterfitt
P.O. Box 3188
West Palm Beach, Florida 33402-3188

NOV 2 7 2007

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West Palm Beach, Florida 33402-3188

NOV 27 2007

PTO/SB/44 (04-05)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

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APPLICATION NO.: 09/830,146

ISSUE DATE : October 5, 2004

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Column 17, line 12, replace "Fraction Ib" with --Fraction IIb--

Column 19, line 51, replace "nitration" with --filtration--

Column 20, line 22, replace "try" with --by--

MAILING ADDRESS OF SENDER (Please do not use customer number below):

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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PTO/SB/44 (04-05)
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APPLICATION NO.: 09/830,146

ISSUE DATE : October 5, 2004

INVENTOR(S) : BEAUDOIN et al.

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Column 20, line 22, replace "try" with --by--

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Akerman Senterfitt
P.O. Box 3188
West Palm Beach, Florida 33402-3188

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (703) 746-4000

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

7590 04/30/2004

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO, on the date indicated below.

Mark D. Passler	(Depositor's name)
	(Signature)
7-29-04	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

TITLE OF INVENTION: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1330	$0	$1330	07/30/2004

EXAMINER	ART UNIT	CLASS-SUBCLASS
WITZ, JEAN C	1651	424-520000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Akerman Senterfitt
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Universite de Sherbrooke

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Canada

Please check the appropriate assignee category or categories (will not be printed on the patent); ☐ individual ☐ corporation or other private group entity ☒ government

4a. The following fee(s) are enclosed:

☒ Issue Fee

☐ Publication Fee

☒ Advance Order - # of Copies 5

4b. Payment of Fee(s):

☒ A check in the amount of the fee(s) is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number 50-0951 (enclose an extra copy of this form).

Director for Patents is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.

(Authorized Signature) (Date) 7-29-04

NOTE; The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

08/05/2004 SHASSEN2 00000137 09830146

01 FC:8001 15.00 OP
02 FC:2501 665.00 OP

TRANSMIT THIS FORM WITH FEE(S)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Beaudoin et al.

Serial No.: 09/830,146 Group Art Unit: 1651

Filed: July 25, 2001 Examiner: Witz, Jean C.

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

TRANSMITTAL LETTER

CERTIFICATE UNDER 37 CFR 1.8(a)
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Reg. No. 40,764

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Please find enclosed for filing:

X PTOL-85B (Issue Fee, Advance Order)
X Check in the amount of $680.00 for issue fee and advance copies.
X Other: One (1) Postcard

Applicant claims small entity status. Please charge any deficiencies or credit any overpayment to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 7-29-04

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
P.O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: 561-653-5000

Docket No. 789-47

{WP191315;1}

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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NOTICE OF ALLOWANCE AND FEE(S) DUE

7590 04/30/2004

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

EXAMINER	
WITZ, JEAN C	
ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 04/30/2004

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

TITLE OF INVENTION: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1330	$0	$1330	07/30/2004

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:
A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

II. PART B - FEE(S) TRANSMITTAL should be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). Even if the fee(s) have already been paid, Part B - Fee(s) Transmittal should be completed and returned. If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

or Fax **(703) 746-4000**

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

7590 04/30/2004

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

TITLE OF INVENTION: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1330	$0	$1330	07/30/2004

EXAMINER	ART UNIT	CLASS-SUBCLASS
WITZ, JEAN C	1651	424-520000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent); ❑ individual ❑ corporation or other private group entity ❑ government

4a. The following fee(s) are enclosed:

❑ Issue Fee

❑ Publication Fee

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s):

❑ A check in the amount of the fee(s) is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

Director for Patents is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.

(Authorized Signature) (Date)

NOTE; The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMIT THIS FORM WITH FEE(S)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

7590 04/30/2004

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

EXAMINER	
WITZ, JEAN C	

ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 04/30/2004

Determination of Patent Term Extension under 35 U.S.C. 154 (b)
(application filed after June 7, 1995 but prior to May 29, 2000)

The Patent Term Extension is 0 day(s). Any patent to issue from the above-identified application will include an indication of the 0 day extension on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Extension is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) system (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (703) 305-1383. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (703) 305-8283.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

Notice of Allowability	Application No.	Applicant(s)	
	09/830,146	BEAUDOIN ET AL.	
	Examiner	Art Unit	
	Jean C. Witz	1651	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *response filed 10/28/03*.

2. ☒ The allowed claim(s) is/are *52-90*.

3. ☒ The drawings filed on *4/20/01* are accepted by the Examiner.

4. ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☒ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☒ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO-1449 or PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application (PTO-152)
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

Jean C. Witz
Primary Examiner
Art Unit: 1651

Issue Classification	Application No.	Applicant(s)	
	09/830,146	BEAUDOIN ET AL.	
	Examiner	Art Unit	
	Jean C. Witz	424	

ISSUE CLASSIFICATION

ORIGINAL CLASS	ORIGINAL SUBCLASS	CROSS REFERENCE(S) CLASS	CROSS REFERENCE(S) SUBCLASS (ONE SUBCLASS PER BLOCK)
424	522	424	523

INTERNATIONAL CLASSIFICATION	
A61K	35/56
	/
	/
	/
	/

(Assistant Examiner) (Date)

Diane Williams 4-25-04
(Legal Instruments Examiner) (Date)

Jean C. Witz
Art Unit 1651
(Primary Examiner) 4/28/04 (Date)

Total Claims Allowed: 39

O.G. Print Claim(s)	O.G. Print Fig.
1	-

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		31	10	61		91		121		151		181
	2		32	11	62		92		122		152		182
	3		33	12	63		93		123		153		183
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	7		37	16	67		97		127		157		187
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	15		45	24	75		105		135		165		195
	16		46	25	76		106		136		166		196
	17		47	26	77		107		137		167		197
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	30	9	60	39	90		120		150		180		210

Search Notes	Application No.	Applicant(s)	
	09/830,146	BEAUDOIN ET AL.	
	Examiner	Art Unit	
	Jean C. Witz	1651	

SEARCHED			
Class	Subclass	Date	Examiner
424	522	4/27/2004	JCW
424	523	4/27/2004	JCW

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner
424	**522**	4/28/2004	**JCW**
424	523	4/28/2004	JCW

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated previous search	4/27/2004	JCW

Index of Claims

Application No.	Applicant(s)
09/830,146	BEAUDOIN ET AL.
Examiner	**Art Unit**
Jean C. Witz	1651

√	Rejected	–	(Through numeral) Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

Claim		Date
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Claim		Date
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Claim		Date
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UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450
www.uspto.gov

MAR 16 2004

J. Rodman Steele, Jr.
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188

In re Application of	:	
Beaudoin et al	:	
Serial No. 09/830,146	:	DECISION ON PETITION
Filing Date: July 25, 2001	:	
Attorney Docket No.: 789-47	:	

This is in response to applicant's "Petition for Acceptance of Date and Mailing of Response to Office Action under 37 CFR 1.8 and/or For Revival of Application for Paten Abandoned Unintentionally under 37 CFR 1.137(b)" filed October 23, 2003. The delay is regretted.

On February 25, 2003, an Office action was mailed. On August 25, 2003, a response with a request for a retroactive extension of time of three months was filed. However, the response was inadvertently addressed to zip code 22313-459, but was clearly addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA.

Applicant's statement and evidence show that a response was filed on August 25, 2003. An extension fee was charged on September 4, 2003 indicating that the Office had received the response. The response is considered to be timely filed. Accordingly, the petition is granted. The instant application has been returned t pending status and will be forwarded to examiner for consideration of the response.

Petition Granted.

Bruce Kisliuk
Director, TC1600

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Beaudoin et al.

Serial No.: 09/830,146 Group Art Unit: 1651

Filed: July 25, 2001 Examiner: Witz, Jean C.

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

REQUEST FOR RETROACTIVE EXTENSION OF TIME

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

on 8-25-03

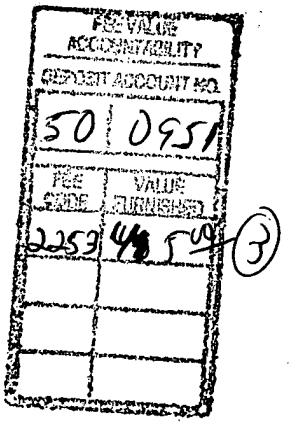
[signature] Reg. No. 40,764

Mail Stop Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1459

FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Dear Sirs:

This is a request for a three month extension of time pursuant to 37 CFR §1.136. Please charge the fee of $465.00, and any deficiencies or credit any overpayments to Deposit Account No. 50-0951.



Respectfully submitted,

Date: 8-25-03

[signature]

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

(WP146887;1)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	09/830,146
Applicant	:	Beaudoin et al.
Filed	:	July 25, 2001
TC/A.U.	:	1651
Examiner	:	Witz, Jean C.
Docket No.	:	789-47
Customer No.	:	30448
Title:		METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

Petition For Acceptance of Date and Mailing of Response to Office Action Under 37 C.F.R. §1.8 and/or For Revival Of Application For Patent Abandoned Unintentionally Under 37 C.F.R. §1.137(b)

CERTIFICATE UNDER 37 C.F.R. §1.8(a)
I hereby certify that this correspondence is being facsimile transmitted to the U.S. Patent and Trademark Office, Fax No. 703-308-6916 on 10-23-03

Reg. No. 40,764.
Mark D. Passler

FAX: 703-308-6916
Attention: Office of Petitions
Commissioner for Patents
Alexandria, VA 22313-1450

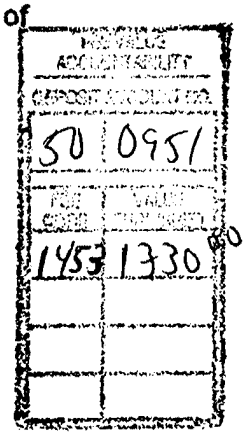
FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Sir:

Applicants hereby petition for revival of the above identified application. Applicant's received a notice that the application has become abandoned due to failure to respond to the Office Action dated February 25, 2003. A copy of Applicant's Response to the Office Action of February 25, 2003 is enclosed.

The Response was mailed to the Commissioner for Patents on August 25, 2003 with a Request for a Retroactive Extension of Time of three months and authorization to charge the appropriate fees therefor to Deposit Account No. 50-0951. The Response was inadvertently addressed to zip code 22313-1459 (as shown by the Certificate of Mailing), but was clearly addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA. The Response was deposited with the U.S. Postal Service with sufficient postage as first class mail and included Mailing Certificates stating the date of deposit for each piece of correspondence.

{WP154915;1}



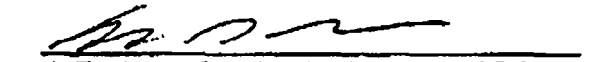
Appln. No. 09/830,146 Docket No. 789-47
Petition in Reply to Notic of Abandonment dated 09/23/2003

The Response is therefore believed to have been received by the U.S. Patent and Trademark Office. In accordance with 37 C.F.R. §1.8, Applicant's request that the Response be deemed to have been timely filed. Applicants thus hereby request that the Response/Amendment is entered, that the notice of Abandonment is withdrawn, and that the above identified application is further examined on its merits.

If the Request for Acceptance of the Date of Mailing Under 37 C.F.R. §1.8 is denied, Applicant's hereby Petition for Revival of the applicant under 37 C.F.R. §1.137(b). Applicants hereby declare that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition under 37 C.F.R. §1.137(b) was unintentional. As stated previously, this paper is accompanied by a copy of the Response to Office Action mailed August 25, 2003. Applicant's request that this response be entered, and that the application is further examined on the merits. Authorization is hereby granted to charge the Petition fee of $1,330.00 to Deposit Account No. 50-0951. As the above referenced application was filed after June 8, 1995, no Terminal Disclaimer and associated fee is believed necessary.

Respectfully submitted,

Date: 10-23-03



J. Rodman Steele, Jr., Reg. No. 25,931
Mark D. Passler, Reg. No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET
(FOR USE WITH FORM PTO-875)

SERIAL NO. 09/830146 | FILING DATE

APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
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TOTAL DEP.			26			
TOTAL CLAIMS			31			

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	IND.	DEP.	IND.	DEP.	IND.	DEP.
51						
52	1					
53		1				
54		1				
55		1				
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88		1				
89		1				
90						
91						
92						
93						
94						
95						
96						
97						
98						
99						
100						
TOTAL IND.	4					
TOTAL DEP.	34					
TOTAL CLAIMS	38					

PTO-1360 (8-98) *MAY BE USED FOR ADDITIONAL CLAIMS OR AMENDMENTS* U.S. DEPARTMENT OF COMMERCE Patent and Trademark Office

PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2000

Application or Docket Number
09/830146

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	34 minus 20=	* 14
INDEPENDENT CLAIMS	5 minus 3 =	* 2
MULTIPLE DEPENDENT CLAIM PRESENT		☐

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐	RATE	FEE	OR	OTHER THAN SMALL ENTITY	RATE	FEE
	BASIC FEE	430	OR		BASIC FEE	
	X$ 9=	126	OR		X$18=	
	X40=	80	OR		X80=	
	+135=		OR		+270=	
	TOTAL		OR		TOTAL	

CLAIMS AS AMENDED - PART II

AMENDMENT A	(Column 1)		(Column 2)	(Column 3)
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	* 45	Minus	** 45	= 0
Independent	* 6	Minus	*** 6	= 0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY	RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY	RATE	ADDITIONAL FEE
	X$ 9=		OR		X$18=	
	X40=		OR		X80=	
	+135=		OR		+270=	
	TOTAL ADDIT. FEE		OR		TOTAL ADDIT. FEE	

AMENDMENT B	(Column 1)		(Column 2)	(Column 3)
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	* 38	Minus	** 45	= —
Independent	* 4	Minus	*** 6	= —
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY	RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY	RATE	ADDITIONAL FEE
	X$ 9=		OR		X$18=	
	X40=		OR		X80=	
	+135=		OR		+270=	
	TOTAL ADDIT. FEE		OR		TOTAL ADDIT. FEE	

AMENDMENT C	(Column 1)		(Column 2)	(Column 3)
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY	RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY	RATE	ADDITIONAL FEE
	X$ 9=		OR		X$18=	
	X40=		OR		X80=	
	+135=		OR		+270=	
	TOTAL ADDIT. FEE		OR		TOTAL ADDIT. FEE	

If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** In the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

FORM PTO-875

Patent and Trademark Office - U.S. DEPARTMENT OF COMMERCE

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	09/830,146
Applicant	:	Beaudoin et al.
Filed	:	July 25, 2001
TC/A.U.	:	1651
Examiner	:	Witz, Jean C.
Docket No.	:	789-47
Customer No.	:	30448
Title:		METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

Transmittal Letter

CERTIFICATE UNDER 37 C.F.R. §1.8(a)
I hereby certify that this correspondence is being facsimile transmitted to the U.S. Patent and Trademark Office, Fax No. 703-308-6916 on 10-23-03

Reg. No. 40,764.
Mark D. Passler

BY FAX: 703-308-6916
Total Pages: 19

Attention: Office of Petitions
Commissioner for Patents
Alexandria, VA 22313-1450

Sir:

Please find enclosed the following for filing:

1. Request for Acceptance of Date of Mailing of Response to Office Action Under 37 C.F.R. §1.8 and/or Petition For Revival Of Application For Patent Abandoned Unintentionally Under 37 C.F.R. §1.137(b)
2. Copy of Response To Office Action Mailed August 25, 2003 including
   (a) Transmittal Letter
   (b) Amendment
   (c) Request for Retroactive Extension of Time
   (d) Copy of Extracts from "Data for Biochemical Research" and "The Merck Index"

If deemed necessary, please charge $1,330.00 for the Petition For Revival of Application Abandoned Unintentionally Under 37 C.F.R. §1.137(b) to Deposit Account No. 50-0951. Please charge any fee deficiencies, or credit any overpayments, to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 10-23-03

J. Rodman Steele, Jr., Reg. No. 25,931
Mark D. Passler, Reg. No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47
(WP154915;1)

RAM Fee History Query

Revenue Accounting and Management

Name/Number: 09830146 Total Records Found: 9

Start Date: Any Date End Date: Any Date

Accounting Date	Sequence Num.	Tran Type	Fee Code	Fee Amount	Mailroom Date	Payment Method
04/25/2001	00000061	1	965	$80.00	04/20/2001	CK
04/25/2001	00000060	1	967	$126.00	04/20/2001	CK
04/25/2001	00000059	1	971	$430.00	04/20/2001	CK
07/26/2001	00000150	1	254	$65.00	07/25/2001	CK
08/01/2001	00000063	1	581	$40.00	07/26/2001	CK
12/06/2002	00000001	1	1251	$110.00	11/20/2002	DA 500951
04/17/2003	00000019	1	1251	-$110.00	11/20/2002	DA 500951
04/17/2003	00000020	1	2251	$55.00	11/20/2002	DA 500951
09/04/2003	00000038	1	2253	$465.00	09/02/2003	DA 500951

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	09/830,146
Applicant	:	Beaudoin et al.
Filed	:	July 25, 2001
TC/A.U.	:	1651
Examiner	:	Witz, Jean C.
Docket No.	:	789-47
Customer No.	:	30448
Title:		METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

P tition For Acceptance of Date and Mailing of Response to Office Action Und r 37 C.F.R. §1.8 and/or For Revival Of Application For Patent Abandoned Unintentionally Under 37 C.F.R. §1.137(b)

CERTIFICATE UNDER 37 C.F.R. §1.8(a)
I hereby certify that this correspondence is being facsimile transmitted to the U.S. Patent and Trademark Office, Fax No. 703-308-6916 on 10-23-03

Reg. No. 40,764.
Mark D. Passler

FAX: 703-308-6916
Attention: Office of Petitions
Commissioner for Patents
Alexandria, VA 22313-1450

FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Sir:

Applicants hereby petition for revival of the above identified application. Applicant's received a notice that the application has become abandoned due to failure to respond to the Office Action dated February 25, 2003. A copy of Applicant's Response to the Office Action of February 25, 2003 is enclosed.

The Response was mailed to the Commissioner for Patents on August 25, 2003 with a Request for a Retroactive Extension of Time of three months and authorization to charge the appropriate fees therefor to Deposit Account No. 50-0951. The Response was inadvertently addressed to zip code 22313-1459 (as shown by the Certificate of Mailing), but was clearly addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA. The Response was deposited with the U.S. Postal Service with sufficient postage as first class mail and included Mailing Certificates stating the date of d posit for each piece of correspondence.

{WP154915;1}

Appln. No. 09/830,146 Docket No. 789-47
Petition in Reply to Notic of Abandonment dated 09/23/2003

The Response is therefore believed to have been received by the U.S. Patent and Trademark Office. In accordance with 37 C.F.R. §1.8, Applicant's request that the Response be deemed to have been timely filed. Applicants thus hereby request that the Response/Amendment is entered, that the notice of Abandonment is withdrawn, and that the above identified application is further examined on its merits.

If the Request for Acceptance of the Date of Mailing Under 37 C.F.R. §1.8 is denied, Applicant's hereby Petition for Revival of the applicant under 37 C.F.R. §1.137(b). Applicants hereby declare that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition under 37 C.F.R. §1.137(b) was unintentional. As stated previously, this paper is accompanied by a copy of the Response to Office Action mailed August 25, 2003. Applicant's request that this response be entered, and that the application is further examined on the merits. Authorization is hereby granted to charge the Petition fee of $1,330.00 to Deposit Account No. 50-0951. As the above referenced application was filed after June 8, 1995, no Terminal Disclaimer and associated fee is believed necessary.

Respectfully submitted,

Date: 10-23-03

J. Rodman Steele, Jr., Reg. No. 25,931
Mark D. Passler, Reg. No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Beaudoin et al.

Serial No.: 09/830,146 Group Art Unit: 1651

Filed: July 25, 2001 Examiner: Witz, Jean C.

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

TRANSMITTAL LETTER

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1459

on 8-25-03

[signature] Reg. No. 40,764

Mail Stop Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1459

FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Sir:

Please find enclosed the following for filing:

- ✓ Amendment
- ✓ Request for Retroactive Extension of Time
- ✓ Copy of Extracts from "Data for Biochemical Research" and "The Merck Index"

Please charge $465 for a three month extension of time plus any fee deficiencies, or credit any overpayments, to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 8-25-03

[signature]

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

(WP146887;1)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Beaudoin et al.

Serial No.: 09/830,146 Group Art Unit: 1651

Filed: July 25, 2001 Examiner: Witz, Jean C.

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

REQUEST FOR RETROACTIVE EXTENSION OF TIME

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1459

on 8-25-03

[signature] Reg. No. 40,764

Mail Stop Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1459

FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Dear Sirs:

This is a request for a three month extension of time pursuant to 37 CFR §1.136. Please charge the fee of $465.00, and any deficiencies or credit any overpayments to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 8-25-03

[signature]

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

(WP146887;1)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Beaudoin et al.

Serial No.: 09/830,146 Group Art Unit: 1651

Filed: July 25, 2001 Examiner: Witz, Jean C.

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

AMENDMENT

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1459

on 8-25-03

[signature] Reg. No. 40,764

Mail Stop Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1459

FAX RECEIVED
OCT 23 2003
PETITIONS OFFICE

Dear Sirs:

This amendment is in response to the Office Action dated February 25, 2003.

Amendments to the claims begin on page 2 of this paper.

Remarks/arguments begin on page 8 of this paper.

{WP146887;1}

AMENDMENTS TO THE CLAIMS

1-51. (Cancelled)

52. (New) A method for extracting total 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 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 total lipid rich fraction from the liquid contents of b) 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 and esters of acetic acid to achieve extraction of the remaining soluble total lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents;

(f) recovering a second total lipid rich fraction by evaporation of the solvent from the liquid contents of e); and

(g) recovering the solid contents.

53. (New) A method as in claim 52, wherein steps (b) and (d) are conducted under inert gas atmosphere.

54. (New) A method as in claim 52, wherein steps (b) and (e) are effected by techniques selected from the group consisting of filtration, centrifugation and sedimentation.

55. (New) A method as in claim 52, wherein steps (c) and (f) are effected by techniques selected from the group consisting of vacuum evaporation, flash evaporation and spray drying.

56. (New) A method as in claim 52, 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).

57. (New) A method as in claim 52, 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).

58. (New) A method as in claim 52, wherein prior to step (a) the marine and aquatic animal material is finely divided.

59. (New) A method as in claim 52, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

60. (New) A method as in claim 52, wherein said marine and aquatic animal is zooplankton.

61. (New) A method as in claim 60, wherein said zooplankton is selected from krill and *Calanus*.

62. (New) A method as in claim 52, wherein said marine and aquatic animal is fish.

63. (New) A method for extracting an astaxanthin-and-canthaxantin-containing total lipid fraction from a marine and aquatic animal material selected from zooplankton and fish, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents; and

(c) recovering a lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents,

whereby an astaxanthin-and-canthaxantin-containing total lipid fraction is obtained.

64. (New) A method for extracting a total lipid fraction from a marine and aquatic animal material selected from zooplankton and fish, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents; and

(c) recovering a lipid rich fraction from the liquid contents by evaporation of the solvents present in the liquid contents,

whereby a total lipid fraction is obtained.

65. (New) A method as in claim 63, wherein the animal material is selected from krill and *Calanus*.

66. (New) A method as in any one of claim 63, wherein during step (a), the animal material is homogenized.

67. (New) A method as in claim 63, wherein steps (b) and (d) are conducted under inert gas atmosphere.

68. (New) A method as in claim 63, wherein step (b) is effected by a technique selected from the group consisting of filtration, centrifugation and sedimentation.

69. (New) A method as in claim 63, wherein step (c) is effected by a technique selected from the group consisting of vacuum evaporation, flash evaporation and spray drying.

70. (New) A method as in claim 63, wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

71. (New) A method as in claim 63, wherein prior to step (a) the marine and aquatic animal material is finely divided.

72. (New) A method as in claim 63, wherein steps (a) and (b) are conducted at solvent temperatures of about 5°C or less.

73. (New) A method of lipid extraction as in claim 52, wherein the solid contents of step b) is recovered and consists of a dehydrated residue containing active enzymes.

74. (New) A method of lipid extraction as in claim 63, wherein the solid contents of step b) is recovered and consists of a dehydrated residue containing active enzymes.

75. (New) A method for extracting total 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 to achieve extraction of the soluble total lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first total lipid-rich fraction from the liquid contents of b) 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 and esters of acetic acid to achieve extraction

of the remaining soluble total lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents; and

(f) recovering a second total lipid-rich fraction by evaporation of the solvent from the liquid contents of e),

whereby total lipid fractions are obtained.

76. (New) A method of total lipid extraction as in claim 75, wherein the solid contents of step b) is recovered and consists of a dehydrated residue containing active enzymes.

77. (New) A method as in claim 52, wherein the ketone solvent is acetone.

78. (New) A method as in claim 52, wherein the alcohol is selected from the group consisting of ethanol, isopropanol and *t*-butanol.

79. (New) A method as in claim 52, wherein the ester of acetic acid is ethyl acetate.

80. (New) A method as in claim 58, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

81. (New) A method as in claim 63, wherein said marine and aquatic animal material is viscera.

82 (New) A method as in claim 63, wherein the ketone solvent is acetone.

83 (New) A method as in claim 64, wherein said marine and aquatic animal material is viscera.

84. (New) A method as in claim 64, wherein the animal material is selected from krill and Calanus.

85. (New) A method as in claim 71, wherein the animal material is finely divided to an average particle size of not more than 5 mm.

86. (New) A method as in claim 52, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

87 (New) A method as in claim 75, wherein the ketone solvent is acetone.

88. (New) A method as in claim 75, wherein the alcohol is selected from the group of ethanol, isopropanol and t-butanol.

89. (New) A method as in claim 75, wherein the ester of acetic acid is ethyl acetate.

90. (New) A method of lipid extraction as in claim 75, wherein the solid contents of step (3) is recovered and consists of a dehydrated residue containing active enzymes.

REMARKS

The foregoing amendments and these remarks are in response to the Office Action dated February 25, 2003. This amendment is filed with a request for a three month extension of time and authorization to charge Deposit Account No. 50-0961 for the extension of time. At the time of the Office Action, claims 1, 4-12, 14-17, 19-27, 30-33 and 37-51 were pending in the application.

I. Claim renumbering and amendments

Reconsideration of this Application and entry of the foregoing amendments are requested. Claims 1, 4-12, 14-17, 19-25, 27, 30-32, 36-44 and 47-51 have been cancelled and new claims 52-90 have been entered. Independent claims 52, 63, 64 and 75 recite a method to extract a total lipid fraction. Support for the amendments to these claims can be found, for example, at page 5, lines 5 to 7 and at page 8, lines 15 to 19. Further support for this terminology may be found throughout the disclosure where it is shown that the lipid fraction extracted from marine and aquatic animal is the total lipid fraction as may be seen more particularly in Table 13. Table 13 describes the various components of the lipids extracted according to the present claims which include triglycerides, free fatty acids, monoglycerides and phospholipids.

Claims 62, 63 and 64 do not recite "filleting-by-products". Support for this deletion may be found in the claims 15 and 16 as originally filed.

II. Rejections Under 35 U.S.C. § 103

The Office Action rejected claims 1, 4-12, 14-17, 19-25, 27, 30-32, 36-44, 47-51 under 35 U.S.C. § 103(a) as being unpatentable over the disclosures of CA 2,155,571, WO 8401715, JP 360035057A and US 6,055,936 ("Collins") taken as a whole.

Prior to discussing the rejections, a brief presentation on the nature and constituents of lipids including those of marine and aquatic animal lipids is provided.

Lipids from animal and plant tissues

Within its generally recognized meaning and within the meaning of that which is recited by the claims, the term "lipid" refers to naturally occurring substances soluble in organic solvents, but insoluble in water. The diverse groups of substances encompassed by this definition can be divided into two broad classes: the acyl lipids and the terpenoids (minor components). The acyl lipids may then be subdivided into further subclasses: neutral acyl lipids (glycerides, free fatty acids and cholesterol esters), and polar lipids including glycerophospholipids, glyceroglycolipids and sphingolipids. Terpenoids are comprised of two subclasses of minor components: the sterols and the chlorophylls and carotenoids. Carotenoids constitute a very minor constituent of lipids in marine animals. The Examiner is referred to Table 17 on page 29 of the present application providing the content in astaxanthin and canthaxanthin of fractions obtained and to page 10, lines 26 to 31 where it is indicated that the content of astaxanthin is about 75-124µg/g and the cont nt of canthaxanthin is about 250-700 µg/g. It is apparent throughout the present application that the term "total lipid" as used therein is meant to encompass all these various types of lipids and that the method of the present invention is able to extract all these various types of lipids.

Marine oils

The oils extracted from marine and aquatic materials can be separated into two types, according to their composition. The oils extracted from most fishes constitute the first oil type. It is composed of more than 90% of neutral acyl lipids, the major part of which are glycerides. Glycerides are fat reserves of marine animals. Cod liver oil for instance, is mostly constituted of glycerides.

The second oil type is composed of a lesser proportion of neutral acyl lipids and of glycerophospholipids, and sphingolipids. Krill lipids for instance may contain about 44% neutral acyl lipids (triglycerides, monoglycerides and free fatty acids) and about 54% of phospholipids (Phosphatidylcholines, Phosphatidylethanolamines, lysophosphatidylcholines

and phosphatidylserines) and sphingolipids (sphingomyelin) (see table 13 of the present application).

Prior to the present invention, the only solvents that appeared to produce good results to extract total lipids from krill was a combination of chloroform and methanol. Notably, however, these solvents are unacceptable for the food industry, because after their evaporation, some toxic residues could remain in the lipids. There is no reported success of total lipids extraction with non-toxic solvents generally recognized as safe (GRAS). Furthermore, although acetone was used to extract certain components of lipids, it was never disclosed to extract total lipids from marine and aquatic animals.

CA 2,155,571, JP 360035057A and Collin

It is respectfully submitted that JP 360035057A, Collin and CA 2,155,571, do not describe the use of acetone or ethanol for extracting "total lipids" within the meaning of the present application but to extract only a small fraction of total lipids, namely carotenoids for JP 360035057A and Collin, and polyunsaturated fatty acids for CA 2,155,571.

JP 360035057A and Collin teach that acetone is a conventional solvent for the recovery of fatty acids and lipid pigments (astaxanthin and canthaxanthin) of marine animals. Both polyunsaturated fatty acids and pigments constitute minor components of marine animal lipids. In the present application, the fraction 1 is a lipid fraction extracted with acetone according to a specific embodiment of the present invention. Analysis of lipid classes shows that free fatty acids constitute about 23.7% (See table 13) of total lipids of that fraction. The pigments, found in the hydrocarbon group (see Table 13) are only present as trace. As may further be seen from Table 17 of the present application, pigments constitute 0.0364% of the total lipid extracts of krill obtained according to the present invention (i.e. 93.1+270.4 =363.5µg/g oil). It is therefore submitted that techniques such as those described in JP 360035057A and Collin, which are able to extract less than 25% of total lipids, cannot be considered to be methods for total lipid extraction.

It is also respectfully submitted that it was not predictable from references such as those describing the extraction of minor components of marine animal lipids, namely

polyunsaturated fatty acids and lipid pigments with acetone, that the remaining major components of lipids could also be extracted from marine animal with acetone. None of the cited references JP 360035057A, Collin or CA 2,115,571 disclose or suggest that acetone can efficiently extract glycerides, glycerophospholipids and sphingolipids from marine animals.

Notably, the cited prior art teaches away from using acetone to extract glycerophospholipids, sphingolipids and glycerides. Dawson et al., *Textbook of RMC*, Third Ed. "Data for Biochemical Research" and *The Merck Index*, 12th edition, Budavari et al., Ed. Merck Research Laboratories, 1996 teach that phosphatidylethanolamines, sphingomyelin and phosphatidylcholine are practically insoluble in acetone and that phosphatidylserine and lysophosphatidylcholine are only slightly soluble in acetone. The Table below presents the solubility of various glycerophospholipids and sphingolipids in acetone as disclosed in these references. Please find enclosed copies of the rel vant extracts of these references.

Lipid	Reference	Solubility
Phosphatidylcholine (lecithin)	Dawson p.181	slightly soluble in acetone soluble in ethanol
	Merck #5452, p.925	insoluble in acetone partially soluble in ethanol
Phosphatidylethanolamines (cephalin)	Dawson p.181	insoluble in acetone soluble in ethanol
	Merck #2022, p.328	insoluble in acetone slightly soluble in ethanol
Phosphatidylserines	Dawson p.183	insoluble in acetone insoluble in ethanol
Phosphatidylinositol	Dawson p.183	insoluble in acetone insoluble in ethanol
Sphingomyelin (sphingosine)	Dawson p.184	insoluble in acetone insoluble in ethanol
	Merck #8899, p.1495	insoluble in acetone insoluble in ethanol

It is also noteworthy that acetone is not mentioned as a potential solvent for triglycerides and that many of these neutral lipids are indicated to be only slightly soluble in ethanol. It is therefore respectfully submitted that at the time of the Applicants' invention, th prior art taught away from using acetone as a solvent for extracting lipids from marine animal. The Applicants themselves fortuitously discovered acetone's potential as a solvent for the extraction of krill lipids while they were investigating for a means to dehydrate krill tissues. In summary, the prior art does not disclose or suggest that the main classes of lipids (glycerophospholipids, sphingolipids and glycerides) can be efficiently extracted with acetone or ethanol.

Destruction of main components of krill lipids

JP 360035057A discloses an process by which krill is combined with an organic solvent such as acetone or hexane and a lipase. The main process seeks to decompose glycerides and phospholipids into fatty acids in order to achieve the concentration of carotenoids which can then be utilized as a dye. Now, as indicated earlier, marin and aquatic animals lipids contain glycerides, free fatty acids, phospholipids, glyceroglycolipids and sphingolipids. Each of these components are constituted in part of fatty acids. Thus, the method of JP 360035057A would damage the major components of marine and aquatic animals lipids.

For medical, cosmetic and nutritional applications, it is advantageous to preserve marine lipid integrity because they are then more easily assimilated in the organism. Hence, phospholipids for instance contribute to the formation of an emulsion with triglycerides during digestion, thereby facilitating lipase action which in turn may progressively release fatty acids and enable their good absorption by the organism. Furthermore, many if not all components of the total lipid extracts have nutritional values. A method able to extract the total lipids is therefore advantageous.

WO 8401715

WO 8401715 describes a carbon tetrachloride (CCl_4) extraction of enzymes from water-extracted lipids (see page 10, lines 4 to 10 of WO 8401715). CCl_4 is toxic according to the Merck Index (#1864, page 297 Merck Index, 12th edition): it may cause liver and kidney damage and may be a carcinogen.

In stark contrast, the method of the present invention is directed to an extraction of total lipids of marine and aquatic animals with a ketone solvent or a mixture of a ketone and an ethanol solvent or ethyl acetate. According to a specific embodiment of the present invention, active enzymes may be recovered from the solid residues produced. WO 8401715 does not disclose or suggest a method for extracting total lipids from marin and aquatic animals with a ketone solvent, the solvent used in WO 8401715 for extracting lipids is CCl_4.

Appln. No. 09/830,146 Docket No. 789-47
Amendment
Response to Office Action dated 02/25/2003

In view of the above and foregoing, withdrawal of the rejection under 35 U.S.C. §103, first paragraph is respectfully requested. The rejections of the original claims are believed to have been overcome by the present remarks and the introduction of new claims. From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order, and such action is earnestly solicited.

III. Conclusion

Applicant has made every effort to present claims which distinguish over the prior art, and it is believed that all claims are in condition for allowance. Nevertheless, Applicant invites the Examiner to call the undersigned if it is believed that a telephonic interview would expedite the prosecution of the application to an allowance. In view of the foregoing remarks, Applicant respectfully requests reconsideration and prompt allowance of the p nding claims.

Respectfully submitted,

Date: 8-25-03

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
AKERMAN SENTERFITT
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

DATA FOR BIOCHEMICAL RESEARCH
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...ear of its sesquicentennial celebration, The Merck Index marks the beginning of its second century of publication. A comparison of this one-volume, 2600-page, multidisciplinary encyclopedia with the thin catalog of Merck products that was published in 1889, underscores the vast changes that have occurred in this publication over twelve editions, and in the realm of scientific information. Although the text continues to be designed primarily to serve the needs of chemists, biochemists, pharmacists, and those in allied professions, it contains information of interest to a broad spectrum of scientists and others in a large number of disciplines. The material is assembled with today's quick reference needs in mind, but the commitment to enhanced scientific communication and to excellence in scientific scholarship remains paramount.

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Susan Budavari, *Editor*
Merck Research Laboratories
Rahway, New Jersey 07065

(London) 1961, 1468. R. W. Thompson in Kirk-Othmer *Encyclopedia of Chemical Technology* vol. 4 (Wiley-Interscience, New York, 3rd ed., 1978) pp 742-759.

Highly reactive, volatile, very flammable liq. *Pancreas*/ The great distillates even obtained are reported to have a reward, pleasing, and mineral odor, while the least unpleasant and reagent grades are foul smelling. Do not standing has a long time. Burns with a blue flame to CO_2 and SO_2. *Acute fire and explosion hazard.* can be ignited by hot steam pipes. Flash pt, closed cup −10°. Ignition pt 190°. Explosion range: 1 to 50% (v/v) in air. d_4^{20} 1.25992, d_4^{25} 1.27015, d_4^{30} 1.28332, d_4^{35} 1.29852. Vapor sank to the ground. Vapor density 3.67 (air = 1). mp −111.6°, bp_{20} −73.8°, bp_{40} −44.7°, bp_{60} −9.1°, bp_{760} +46.0°; bp_{20} 106.1°; bp_{40} 68.1°, bp_{60} 41.8°. Dry temp 310.0°, wet temp 72.9 atm. $n_D^{-11.6}$ 1.45759, n_D^{0} 1.43803, n_D^{11} 1.42943. Surface tension at 20° 31.75. Coefficient of viscosity at 20° 0.363. Heat of vaporization at bp, 84.1 cal/g. Heat of fusion 1.049 kcal/mole. Heat capacity at 24.7° 18.71 cal/mole/deg. Burns. Mauser, *J. Am. Chem. Soc.* 99, 5000 (1977). Bimolecular constant, 2.35°. Dielectric constant at low frequencies, 2.641. Dipole moment: 0.0 Soly in water at 20° 0.294%. Soly of water in CS_2: <0.0055. Azeotrope with water bp 42.6°, contains 97.2% CS_2. Miscible with anhydr methanol, ethanol, ether, benzene, chloroform, carbon tetrachloride, oils. Can be stored in iron, aluminum, glass, porcelain, Teflon.

Caution: Poisoning usually occurs from inhalation but also may be caused by ingestion and skin absorption. *Acute Toxicity:* euphoria, excitement, nervous membrane irritation, nausea, vomiting, unconsciousness, terminal convulsions. *Chronic Toxicity:* marked psychic disturbances ranging from nervous irritability to severe with hallucinations, tremors, auditory and visual disturbances, weight loss, blood dyscrasias. Disease caused with carbon may cause burning pain, erythema, exfoliation. *See Clinical Toxicology of Commercial Products* R. E. Gosselin *et al.* Eds (Williams & Wilkins, Baltimore, 4th ed., 1976) Section III, pp 83-86.

Use: In the manufacture of rayon, carbon tetrachloride, semiorganics, and disinfectants, electronic vacuum tubes. Solvent for phosphorus, sulfur, selenium, bromine, iodine, fats, resins, rubbers.

1860. Carbonic Anhydrase. *Carbonate dehydratase; carbonate hydro-lyase.* Mol wt ~30,000. A small zinc-containing enzyme which catalyzes the hydration of CO_2. Found in higher mammals in erythrocytes, renal cortex, and gastric mucosa of mammals; also found in other animal tissues, in plant and in some bacteria. Isolated from bovine erythrocytes. Lundsgaard, *Biochem. Biophys. Acta* 19, 218 (1940). From human erythrocytes: Nyman, *Acta* 53, 1 (1961). From renal cortex: Hibbs, *Proc. Soc. Exp. Biol. Med.* 69, 67 (1948). From gastric mucosa: Davenport, *Physiol. Rev.* 24, 360 (1944). Human carbonic anhydrase consists of two isoenzymes with distinctly different amino acid sequences and specific activities. The high activity form is called carbonic anhydrase C; the low activity form is called B, modified forms of these two isoenzymes exist. Fernandez, Deutsch, *J. Biol. Chem.* 243, 6474 (1968); 244, 3458 (1969). Amino acid sequence of carbonic anhydrase B: Anderson *et al.*, *Biochem. Biophys. Res. Commun.* 48, 690 (1972); Lin, Deutsch, *J. Biol. Chem.* 248, 1847 (1973). sequence of carbonic anhydrase C: Henderson, Henriksson, *Biochim. Biophys. Acta Commun.* 57, 1308 (1975); Lin, Deutsch, *J. Biol. Chem.* 249, 2378 (1974). Crystal structure of carbonic anhydrase C: Liljas, *et al.*, *Nature New Biol.* 235, 131 (1972). Catalyzes the reversible reaction of CO_2 and H_2O to HCO_3^- and H^+. Provides CO exchange between blood and tissues. In gastric mucosa, provides ions in addition to ions to facilitate the excess alkalinity produced by the secretions of water and excretion of hydrogen ion. Roughton, Clark in *The Enzymes* vol. 2, part 2, J. B. Sumner, K. Myrbäck, Eds. (Academic Press, New York, 1951) pp 1253-1265. In the kidney, participates in the transport. Review of physiology: 64 refs in *Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status, Alfred Benzon Symposium IV*, P. Astrup, M. Rørth, Eds. (Munksgaard, Copenhagen, 1972) pp 416-42. Review of mechanism of reaction: Prince, Woolley, *Angew. Chem. Int. Ed.* 10, 408-417 (1971). Review: Lindskog *et al.*, "Carbonic Anhydrase" in *The Enzymes* vol. 4, P. D. Boyer, Ed. (Academic Press, New York, 1971) pp 587-665.

1861. Carbon Monoxide. CO. mol wt 28.01. C 42.85%, O 57.13%. Produced on an industrial scale by partial oxidation of hydrocarbon gases from natural gas or by the gasification of coal and coke. Conveniently prepared in the laboratory by heating calcium carbonate with Zn dust. Whitehouse, *J. Am. Chem. Soc.* 70, 442 (1948); by dehydration of formic acid with H_2SO_4. Gilliland, Blanchard, *Inorg. Syn.* 2, 61 (1946). Purification of carbon monoxide bought in steel cylinders: A. Klemenc, *Die Behandlung und Reinigung von Gasen* (Vienna, 1949) p 160. Glemser in *Handbook of Preparative Inorganic Chemistry* vol. 1, G. Brauer, Ed. (Academic Press, New York, 2nd ed., 1963) p 444. Review of toxic effects in humans: Stewart, *Ann. Rev. Environ.* 2, 409-420 (1975). Review: C. M. Brenda, G. M. Dripps in Kirk-Othmer *Encyclopedia of Chemical Technology* vol. 6 (Wiley-Interscience, New York, 3rd ed., 1978) pp 772-795.

Highly poisonous, odorless, colorless to violet gas. Very flammable, burns in air with a bright blue flame. Ignites in hot air. 1007. mp −203.0°. bp −191.5°. d_4^{-205} (liq) 0.814, d (gas) 0.968 (air = 1.000). d_4^{0} at 760 mm: 1.250 g/liter. The vap pressure is 5500 psi. Flammable limits in air: 12 to 75 vol %. Crit pressure 35 atm, crit temp −139°. Heat capacity at 20°: 6.91 cal/mole/°C. Heat value per mol: 3033 kcal. Heat of formation −26.39 kcal/mol. Dec into carbon and carbon dioxide between 400 and 700°, at lower temp when in contact with catalysts: sunlight. Above 800° the equilibrium reaction favors CO formation. Hopcalite, a mixture of the oxides of manganese and copper, catalyzes the decomp at room temp, as does Pd on silica gel. Sparingly sol in water: 3.3 cc/100 cc H_2O at 0°, 2.3 cc/100 cc H_2O at 20°. Readily absorbed by ammoniacal soln of cuprous chloride in HCl or in NH_4OH. Appreciably sol in some organic solvents, such as ethyl acetate, $CHCl_3$, acetic acid. The only known alcohol and ethanol is about 7 times as great as the solubility in water.

Caution: Combines with the hemoglobin of the blood to form carboxyhemoglobin, which so reduces the oxygen carrying. *Toxic symptoms:* Headache, mental dullness, dizziness, weakness, nausea, vomiting, loss of muscular control, increased then decreased pulse and respiratory rates, collapse, unconsciousness, death. *Antidote:* Oxygen. *See Patty's Industrial Hygiene and Toxicology* vol. 2C, G. D. Clayton, F. E. Clayton, Eds. (Wiley-Interscience, New York, 3rd ed., 1982) pp 4114-4126.

Use: As reducing agent in metallurgical operations especially in the blast process for the recovery of metal, in organic synthesis especially in the Fischer-Tropsch process for petroleum-type products and in the oxo reaction in the manufacture of metal carbonyls.

1862. Carbon Nitride. C_3N_4. mol wt 92.04. C 39.18%, N 60.86%. Crystalline solid which exists in two forms, α and β. β-form may be harder than diamond. Model of structure and hardness: A. Y. Liu, M. L. Cohen, *Science* 245, 841 (1989). Synthesis: L. E. Hallis *et al.*, PCT Int. pat. Appl. WO 91 16,166, cited, U.S. pat. 5,110,679 (1992, 1992 both to Regents U. California, Berkeley). C. Niu *et al.*, *Science* 261, 334 (1993). Hardness evaluation: D. Fakunaga, *Kagaku to Kogyo* 45, 321 (1992). C.A. 119, 245784 (1993).

1863. Carbon Suboxide. 1,3-Propadiene-1,3-dione, tetracarbon dioxide. C_3O_2. mol wt 68.03. C 52.96%, O 47.06%. O=C=C=C=O. Preps by thermal decomp of squaric acid: Gleiter in *Handbook of Preparative Inorganic Chemistry* vol. 2, G. Brauer, Ed. (Academic Press, New York, 2nd ed., 1963) p 645. Reactions in organic synthesis: Diels-Alder, Beitr. *Rost Chem. Rev.* 24, 391 (1967). Comprehensive review: Reynolds, *Chim. Chem. Rev.* 7, 177 (1980); Vollhardt, *Angew. Chem.* 6, 610 (1967); Gleiter, *Chimia* 28, 11 (1980); T. Kappe, E. Ziegler, *Angew. Chem. Int. Ed.* 13, 491-501 (1974); reprinted in *Adv. Heterocyclic Chem.* 18, 1 (Verlag Chemie, Weinheim, 1976) pp 2949.

Colorless, highly refractive liquid or colorless gas which burns with a blue, sooty flame. Odor like acrolein and unsaturated. mp −111.3°. bp_{760} 6.8°. d_4^{0} 1.114. n_D^{0} 1.45384, n_D^{20} 1.44739. Vapor pressure at 0° 357-389 mm. Explosive limits: 6 to 30 vol % in air. Dipole moment: 0.7D. Thermodynamic constants: Thompson, *Trans. Faraday Soc.* 57, 249 (1961). Thin gas can be stored at pressures of up to 180 mm, but even at these pressures polymerization may occur, giving a red, water-sol product. Thin reportedly occurs at higher pressure or in the liquid state. Polymerization facilitated by presence of P_2O_5. Dec when passed through heated glass tubes, leaving a mirror surface. Differentiation of carbon dioxide, ethene. With water forms malonic acid quantitatively. Forms malonamide with ammonia.

Caution: In small amounts acts as a lacrimator, in high concn irritates eyes, nose, respiratory organs, producing a feeling of suffocation.

Use: Preps of malonates, improving dye affinity of fibers.

1864. Carbon Tetrachloride. *Tetrachloromethane; perchloromethane; Necatorina; Benzinoform.* CCl_4. mol wt 153.82. C 7.81%, Cl 92.19%. Obtained from carbon disulfide and chlorine in presence of a catalyst, e.g., $SbCl_3$, Fe filings or by the chlorination of hydrocarbons. Fields, Kreps & Clark's *Industrial Chemicals* F. A. Lowenheim, M. K. Moran, Eds. (Wiley-Interscience, New York, 4th ed., 1975) pp 330-336. H. D. Dellaw in Kirk-Othmer *Encyclopedia of Chemical Technology* vol. 5 (Wiley-Interscience, New York, 3rd ed., 1979) pp 704-714. Toxicity data: Switzerland. *J. Ind. Hyg. Toxicol.* 29, 387 (1947). E. Browning, *Toxicity and Metabolism of Industrial Solvents* (Elsevier, New York, 1965) pp 173-188. Use in evaluation of experimental liver disease: P. Tavoloni, A. P. Meserol, *Br. J. Exp. Pathol.* 64, 35 (1983). Review of carcinogenic risk: *IARC Monographs* 20, 371-399 (1979), of toxicity studies: R. D. Rosenlund *et al.*, *Pharmacol. Ther.* 43, 139-154 (1989).

Colorless, clear, noncflammable, heavy liquid, characteristic odor. May form phosgene when used to put out electrical fires. *Use only when adequate ventilation is provided.* d_4^{25} 1.589, bp 76.7°, mp −23°, n_D^{20} 1.4601. One ml dissolves in 2000 ml water, mix with alcohol, benzene, chloroform, ether, carbon disulfide, petroleum, oils. LC_{50} in rats: 9526 ppm (4-hr/hr). LD_{50} in rats, misc. dogs (g/kg): 2.82, 11.1-14.4, 2.3 orally; LD_{50} in mice (g/kg): 4.1 ip, 20.4 s.c. (IARC, 1979).

Caution: Poisoning may occur by inhalation, ingestion or skin absorption. Acute exposure to high concentrations results in depression of the central nervous system manifested as loss of consciousness, dizziness, vertigo, headache, depression, mental confusion, nervousness, and gastrointestinal disturbances such as nausea, vomiting, abdominal pain and diarrhea. Acute exposure may result in renal damage, leading to anuria and azotemia, and liver injury. Chronic exposure with liver and kidney damage. Skin contact can lead to dermatitis through defatting action. Inhalation may cause pulmonary edema and alveolitis. Consumption intake with significant amounts of alcohol increases probability of injury. *See Patty's Industrial Hygiene and Toxicology* vol. 2B, G. D. Clayton, F. E. Clayton, Eds. (Wiley-Interscience, New York, 3rd ed., 1981) p 3472-3478; *Clinical Toxicology of Commercial Products*, R. E. Gosselin *et al.*, Eds (Williams & Wilkins, Baltimore, 5th ed., 1984) Section III, p 101-107. This substance may reasonably be anticipated to be a carcinogen. *Seventh Annual Report on Carcinogens* (PB85-105781, 1984) p 117.

Use: As solvent for oils, fats, lacquers, varnishes, rubber cements, stating material in manuf of organic compds (esp halogen). Pharmaceutic aid (solvent). Formerly used as dry cleaning agent and fire extinguishing.

Trade use: Formerly as anthelmintic (Necatorina).

Trade use (vet): Anthelmintic.

1865. Carbon Tetrabromide. *Tetrabromomethane; Taxos 14.* CBr_4. mol wt 331.63. C 3.63%, Br 96.37%. Prepd from carbon or carbon monoxide and bromine: Vorl, *Inorg. Syn.* 3, 24 (1950); Bennett, Black, *J. Am. Chem. Soc.* 65, 2043 (1939). Reviewed in *Handbook of Preparative Inorganic Chemistry* vol. 1, G. Brauer, Ed. (Academic Press, New York, 2nd ed., 1963) p 220. May also be prepd from InC_4 + Fe_2: Prout, *Inorg. Syn.* 3, 178 (1950).

Colorless, odorless gas. Thermally stable. Chemically very inert. d (solid, −195°) 1.98. d (liq, −183°) 1.89. mp −183.6°. bp −127.8°. May be stored in steel cylinders.

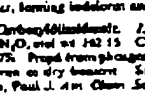
Caution: Narcotic in high concn.

Use: Low temp refrigerant, gaseous insulator.

1866. Carbon Tetraiodide. *Tetraiodomethane.* CI_4. mol wt 519.63. C 2.31%, I 97.69%. Prepd by the interaction of carbon tetrachloride and aluminum or calcium iodide: Gustavson, *Ann.* 172, 171 (1874), Bowen Iodide Mission, *Compt. Rend.* 113, 190 (1891). Ishaom or calcium iodide: Lantzsch, *ibid* 856, 1365 (1953)), ethyl iodide on the presence of aluminum chloride: Walter, *J. Chem. Soc.* 85, 1090 (1904). Modification: Sarzeau, *Inorg. Syn.* 5, 37 (1950).

Red cubic crystals. Odor of iodine. Dec on intense and intermediate ultraviolet light in the presence of air to form I_2 and CO_2. Sol in benzene, chloroform. Dec by hot alcohol. Practically insol in water, but hydrolyzes slowly in contact with water, forming iodoform and iodates.

1867. *N,N'*-Carbonylbisimidazole. *1,1'-Carbonylbisimidazole.* $C_7H_6N_4O$. mol wt 162.15. C 51.85%, H 3.73%, N 34.55%, O 9.87%. Prepd from phosgene and imidazole in dry benzene/toluene or dry hexane: Staab, *Ann.* 609, 75 (1957); Anderson, Paul, *J. Am. Chem. Soc.* 80, 4423 (1958).



Crystals from benzene/diethyl ether, mp 119.5-121°. Should be handled under exclusion of atmospheric moisture. Hydrolyzed by water at a fair rate with evolution of CO_2.

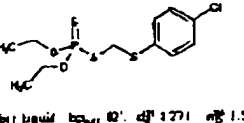
Use: In the synthesis of peptides. Reacts readily with carboxylic acids to form acyl imidazoles, subsequent reaction with amines to form amides goes smoothly.

1868. Carbonyl Fluoride. *Carbonic difluoride, fluorophosgene.* CF_2O. mol wt 66.01. C 18.20%, F 57.56%, O 24.24%. COF_2. Prepd from CO and F_2 at 300°, and CO: Ruff, Mühlenbruch, *Z. Anorg. Allgem. Chem.* 231, 154 (1935). Reviewed in *Handbook of Preparative Inorganic Chemistry*, vol. 1, G. Brauer, Ed. (Academic Press, New York, 2nd ed., 1963) p. 206. Alternate route from CO + AgF_2: Fowler *et al.*, *Inorg. Syn.* 6, 155 (1960).

Pungent, very hygroscopic gas. d (solid, −197°) 1.388, d (liq, −114°) 1.139, mp −114.5°, bp −83.1°. Heat of formation 166.6 kcal. Violently hydrolyzed by water.

Caution: A strong irritant to skin, eyes, mucous membranes, respiratory tract.

1869. Carbophenothion. *Phosphorodithioic acid S-[[(4-chlorophenyl)thio]methyl] O,O-diethyl ester; S-[(p-chlorophenylthio)methyl] O,O-diethyl phosphorodithioate; O,O-diethyl S-(p-chlorophenylthiomethyl) phosphorodithioate.* R-1303. Geigy trade. Trithion. $C_{11}H_{16}ClO_2PS_3$; mol wt 342.87. C 38.53%, H 4.70%, Cl 10.34%, O 9.33%, P 9.03%, S 28.06%. Prepn: Raecker, U.S. pat 2,780,736 (1957 to Geigy). Toxicity study: T. B. Gaines, *Toxicol. Appl. Pharmacol.* 14, 515 (1969).

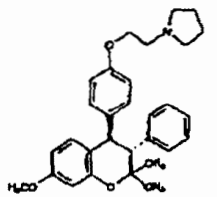


Light amber liquid. $bp_{0.01}$ 82°. d_4^{25} 1.271. n_D^{25} 1.5970, n_D^{30} 1.5898 (Forschirn). Very low vapor pressure. Practically insol in water. Miscible with vegetable oils and most organic solvents. LD_{50} in female, male rats (mg/kg): 10, 10 orally; 27, 54 dermal by (Gaines).

Caution: Cholinesterase inhibitor.

Use: Miticide; insecticide.

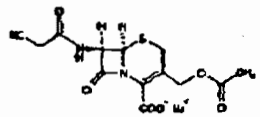
1870. Carboplatin. *(SP-4-2)-Diammine[1,1-cyclobutanedicarboxylato(2-)-O,O']platinum; 1,1-cyclobutanedicar-*



Crystals from ether/petroleum ether, mp 99-101°, solubility ~50. Hydrochloride, $C_{20}H_{28}NO_2$ HCl, *67138 CDRE*. White crystals, mp 165-166° (dec) (methanol). 237, 273 nm (ε 3704 at 278 nm). pKa 2.1. Sol in 60 parts chloroform, 20 parts acetone, 40 parts 95% ethanol. 20 parts methanol. Practically insol in water, isobutanol. 0.1 N HCl. 0.1 N NaOH. LD_{50} ip in mice: 460 mg/kg (Chiu).

THERAP CAT: Antineoplastic.

2016. Cephacetrile Sodium. *(6R-trans)-3-[(Acetyloxy)methyl]-7-[(cyanomethyl)amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monosodium salt; sodium 7-(D-cyanoaminoacetamido)-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate acetate (ester); sodium 7-D-cyanoglycylamido)cephalosporanate acid.* CAS 38278-Ba; Cefaspor; Vezimaze. $C_{14}H_{15}N_4NaO_6S$, mol wt 390.35. C 43.22%, N 13.33%, Na 5.68%, O 24.67%, S 8.17%. Semi-synthetic cephalosporin antibiotic. Prepn: Neth. pat. Appl 6,604,364, Bird *et al*, U.S. pat. 3,483,197 (1969, 1969 both to Ciba). *Antibiotic activities:* Kamal *et al*, *Antibiotics Ag Chemother* 1970, 140; Nou *et al*, *J Antibiot* 23, 480 (1970). Pharmacokinetics and clinical results: Kradolfer *et al*, *Schweiz Med Wochenschr* 102, 311 (1972); Rau *et al*, *Ibid* 716. Toxicology: Kradolfer *et al*, *Arzneimittel Ag Chemother* 1970, 250. *Review:* Arzneimittel-Forsch. 24, 1446-1533 (1974).



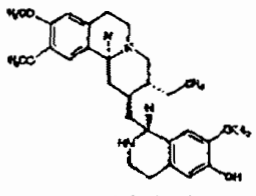
White crystalline powder. LD_{50} in mice (mg/kg): 4500 ± 340 (s.c.), 1100 ± 1500 (i.v.) (Kondo *et al*).

Fine, needle-like crystals from acetone + ether, mp 161-170° (dec). uv max in 0.1 N $NaHCO_3$: 260 nm (ε 9500).

THERAP CAT: Antibacterial.

THERAP CAT (VET): Antibacterial.

2016. Cephalexin. *7-[(R)-1-Aminocyclohexanecarboxamido]-3-methyl-3-cephem-4-carboxylic acid monohydrate; 7-(D-α-aminocyclohexanecarboxamido)-3-methyl-3-cephem-4-carboxylic acid monohydrate.* $C_{16}H_{17}N_3O_4S$, mol wt 466.61. C 72.07%, H 6.91%, N 6.03%, O 13.72%. Next to cefazolin the most important antibiotic of Lilly, the ground work of Umezaga (*see* Doyle) built (*Cephalosporium sporonatum* (Brot.) A. Roch.). *References:* Chen, Pyman, *J Chem Soc* 105, 1591 (1914). *Hinsberg, Ann* 403, 1 (1914). *Structure:* Pallos, Pierrebourg, *Monatsh* 98, 801 (1967). Biotransformation: Van Tamelen *et al*, *J. Am. Chem Soc* 79, 4817 (1957). Partial synthesis: *A. K. Craig, J. R. Corr, Tetrahedron Letters* 50, 4321 (1969). Biosynthetic studies: cited, *Ibid* 1968, 141. HPLC: *Gutierrez* in Biological Stds: D. J. Crouch *et al*, *J Anal Toxicol* 6, 63 (1964). *Review:* M. Sassa in Morazzi, Scriban, *The Alkaloids* vol 3, (Academic Press, New York, 1953) pp 363-394.



Needles from ethanol. Partially bitter taste. mp 115-118° ($[\alpha]_D^{20}$ = +13.6° ± 1° in chloroform). Practically equal in water. Freely sol in dil hydrochloric acid, dil sulfuric and acetic acid, methanol, ethanol, acetone, chloroform. Less sol in ether, petr ether.

Dihydrochloride heptahydrate. $C_{20}H_{28}Cl_2N_2O_2 \cdot 7H_2O$, prisms, sinters at 245°, slowly melts at 270° ($[\alpha]_D^{20}$ +15.0° (c = 2) in water. Solns very yellow. Less sol in alcohol, acetone, chloroform. Practically insol in benzene, hexane.

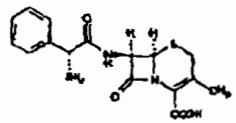
Dihydriodide dihydrate. $C_{20}H_{28}N_2O_2 \cdot 2HI \cdot 2H_2O$, prisms from dil hydrobromic acid, sinters at 240°, slowly melts up to 255°. Sol in water, moderately sol in alcohol, acetone. Practically insol in benzene.

Methyl ether. *See* Sevedrine.

THERAP CAT: Emetic, antiamebic.

THERAP CAT (VET): Has been used as an emetic and expectorant.

2018. Cephaloin. *[6R-[6α,7β(R*)]]-7-[(Aminophenylacetyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; 7-(D-α-aminophenylacetamido)-cephalosporanic acid; 7-(D-2-amino-2-phenylacetamido)-3-methyl-Δ³-cephem-4-carboxylic acid.* Cefadrox, Cefalotin, Cefanex, Cefaspor, Cefotrexine, Dri; Duracef, Eli Jura, Fasgen; Forgen 500, Glenam, Hikta; Ivadrin; Lorcan, Lexidrone, Lincocin; Medimide; Monalexin; Mondrin, Obliksin; Oncocin, Rocef; Sefrocephalin; Sefrokapa, Sefril, Tomefrin, Velosporin, Xafil. $C_{16}H_{17}N_3O_4S$, mol wt 347.39. C 55.32%, H 4.93%, N 12.10%, O 18.42%, S 9.23%. Semi-synthetic cephalosporin antibiotic. Preps: B. R. Morin, E. C. Jackson, U.S. pats. 3,275,626; 3,307,861 (1966, 1970 both to Lilly). Ryan *et al*, *J. Med Chem* 12, 300 (1969). Pharmacology and toxicology: Slaughter *et al*, *Ann marsch Ag Chemother* 1966, 353; Kind *et al*, *Wiener med. Mod* 361, 419. Comprehensive description: L. P. Hamdi, *Anal Profiles Drug Subs.* 4, 21-46 (1975). Clinical pharmacology, efficacy, adverse reactions: *Postgrad Med J* 48, *Suppl* 3, 8-56 (1972).



Crystals, uv max: 260 nm (ε 7250). pK_{a1} 5.2, 7.3.

Monohydrate. *Cefadroxil, Cefamedar, Duricef, Oracefal, Oracefin, Ospexin, Reflex, Roxfin, Ultracef, Unicephalin, Sumacrom, Syncrom.* LD_{50} ip mice, rats (g/kg): 1.6-4.5, > 5.0; orally, rats 1.1, > 2.2 g (Willits).

Monohydrochloride monohydrate. $C_{16}H_{17}N_3O_4S \cdot HCl \cdot H_2O$, LY-001168, Reflex.

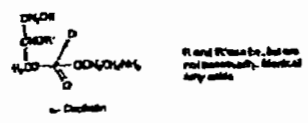
Sodium salt, $C_{16}H_{16}N_3NaO_4S$. *Alfaspoxin.*

Potassium salt. *See* Potacillin.

THERAP CAT: Antibacterial.

THERAP CAT (VET): Antibacterial.

2017. Cephalins. *Kephalins;* phosphatidylethanolamines. Group of phospholipids found in all living organisms. Significant constituent of nervous tissue and brain substance. Cephalins consist of glycerophosphoric acid in which the two free hydroxyls are esterified with long-chain fatty acid residues, and a hexahydric alcohol forms an ester linkage with the phosphoric group. α-Isomers are derivatives of α-glycerophosphoric acid, q.v.; β-isomers are derivatives of β-glycerophosphoric acid, q.v. Natural products occur in the α-form, while the β-form is now recognized to be an artifact. Preps from commercial "soybean lecithin": Schönfeld, Dertinger, *Biochem Prepn* 5, 5 (1957). U.S. pat. 2,801,255 (1957 to USDA). Diagnostic use: C. R. Ratliff *et al*, *Am J Gastroenterol* 56, 369 (1971). Reviews on natural and synthetic cephalins: E. Baer in *Progress in the Chemistry of Fats and Other Lipids* vol. 1, (MacMillan, New York, 1963) pp 39-66, Van Deenen, de Haas in *Advances in Lipid Research*, vol. 1, (Academic Press, New York, 1964) pp 183-189, Verkleij, *Biol Soc Chim Pharm* 1969, 1933.



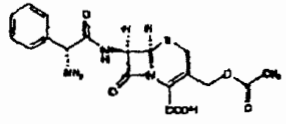
R and R' may be fatty acid residues, identical or different

Yellowish amorphous substances, characteristic odor and taste. Practically insol in water, acetone. Freely sol in chloroform, ether, slightly sol in ethanol.

USE: Clinical reagent (Bruce Junction test).

THERAP CAT: Hemostatic (Good).

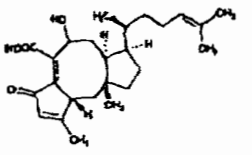
2023. Cephalexin. *[6R-[6α,7β(R*)]]-3-[(Acetyloxy)methyl]-7-[(2-amino-2-phenylacetyl)amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; 7-(2-amino-2-phenylacetamido)-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid acetate (ester); 7-(D-2-amino-2-phenylacetamido)-3-acetoxymethyl-Δ³-cephem-4-carboxylic acid, 3-deacetoxycephalexin, 3-acetoxymethyl-7-(D-α-phenylglycylamido)-Δ³-cephem-4-carboxylic acid monohydrate.* Cephalexin monohydrate, Keflex, Keflet, Keflex, Oracef, Ospexin, Sefrocen, Sporidex, Syncef. $C_{16}H_{17}N_3O_4S$; mol wt 347.39. C 55.32%, H 4.93%, N 12.10%, O 18.42%, S 9.23%. Semi-synthetic cephalosporin antibiotic. Preps: B. R. Morin, E. C. Jackson, U.S. pats. 3,275,626; 3,307,861 (1966, 1970 both to Lilly). Ryan *et al*, *J. Med Chem* 12, 300 (1969). Pharmacology and toxicology: Slaughter *et al*, *Ann marsch Ag Chemother* 1966, 353; Kind *et al*, *Wiener med. Mod* 361, 419. Comprehensive description: L. P. Hamdi, *Anal Profiles Drug Subs.* 4, 21-46 (1972). Clinical pharmacology, efficacy, adverse reactions: *Postgrad Med J* 48, *Suppl* 3, 8-56 (1972).



Dihydrate. *Refocin.* Crystalline powder, mp 273-280° (dec). uv max (2% DMF): 251 nm ($E_{1cm}^{1\%}$ 365). Isoelectric point and pH of maximum stability: 4.5.

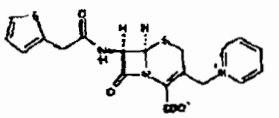
THERAP CAT: Antibacterial.

2024. Cephalonic Acid. *6-Hydroxy-3-cephalosporin-3,7-[6,7-trans-]dihydroxy-3-heptenoic acid;* cephalosporin D. $C_{16}H_{21}O_7$; mol wt 453.34. C 56.99%, H 9.80%, O 33.95%. Minor antibiotic metabolite produced by *Cephalosporium acremonium*. Exhibits weak activity against *Staphylococcus aureus*. Isolation, structure elucidation and physical data: Abraham *et al*, *Proc. Roy. Soc. London Series* 1967, 4411; Abraham *et al*, *Ibid* 4113. Crystal structure: Bat *et al*, *Acta Cryst* 26B, 612 (1969). Synthesis: *Studies on related compounds:* P. C. Dutia, T. R. Das, *Synth Commun* 4, 251 (1974), W. G. Dauben, D. S. Man, *J Org Chem* 42, 912 (1977), R. K. Boeckman *et al*, *Ibid* 3630.



Solid, mp 139°. $[\alpha]_D$ +76.7° (c = 0.54 in chloroform). uv max: 250 nm (ε 11700).

2025. Cephaloridine. *(4R-trans)-1-[[7-(Carboxy)-8-oxo-7-[(3-(4-(aminocarbonyl)-1-pyridinio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]pyridinium-2-carboxylate, Δ²-[7-[(2-thienyl)acetamido]-cephem-3-ene-3-yl-methyl]-pyridinium-4-carboxylate, N-[7-(2-thienylacetamido)-3-ene-3-ylmethyl]pyridinium 2-carboxylate, cefaloridin, Cefaxon, Cephaloridin, Ceri, Fortaxime, Caperon, Caperin, Ofilac, Loridine, Kefloridin, Kefloridin (chloride), Lilacetal; Ledaxin, Cepadrox, Duflorin, Keflopon, Lorafina, Amplyrum, Floridin.* $C_{19}H_{17}N_3O_4S_2$; mol wt 415.49. C 54.93%, H 4.12%, N 10.11%, O 15.40%, S 15.44%. Semi-synthetic cephalosporin antibiotic. Preps: Eli, pat. 3,304,197 (1965 to Glaxo), *CA* 63, 11398c (1965). E. H. Flynn, U.S. pat. 3,488,350 (1969 to Lilly). Toxicity studies: Atkinson *et al*, *Toxicol Appl Pharmacol* 8, 396 (1966).



Crystals. Soluble in water. Aq solns are slightly acid (pH 4.5-5), $[\alpha]_D$ +47.7° (c = 1.75 in water). uv max: 239, 257 nm (ε 15400, 13900) pKa 2.2. Aq solns (10% wt/v) are stable for four weeks at 4°C the deal. Exposure to sunlight causes discoloration. LD_{50} mice, rats (g/kg): > 15, 2.5-4 orally, in animals (g/kg): > 0.2 s.c. (Atkinson).

THERAP CAT: Antibacterial.

THERAP CAT (VET): Antibacterial.

2026. Cephalosporin C. *[6R-[6α,7β(R*)]]-3-[(Acetyloxy)methyl]-7-[(5-amino-5-carboxy-1-oxopentyl)amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; 7-(5-amino-5-carboxypentanamido)-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid acetate.* $C_{16}H_{21}N_3O_8S$; mol wt 415.42. C 46.26%, H 5.10%, N 10.12%, O 30.81%, S 7.72%. Antibiotic substance produced along with penicillin N by a *Cephalosporium* sp. cultivated from sea water and a sewage outlet on the coast of Sardinia. G. Brotzu, *Lav Ist Igiene Sassari* (1948). Newton, Abraham, *Nature* 175, 548 (1955); *cidium Antibiot J.* 62, 651 (1956), *idem, Gen. pat.* 1,004,281 (1957 to Nat Res Dev Corp.), Brit. pat. 818,306 (1959), Kell *et al*, U.S. pat. 3,085,355 (1963 to Nat Res Dev Corp.), Demain, U.S. pats. 3,168,746,77 (1963 to Merck & Co.). Biosynthesis: Demain, Newlands, *Appl Microbiol* 10, 321 (1962). Has the same side chains as penicillin N, but the positions is different and it contains 7-aminocephalosporanic acid. Jago, Huxley, *Brit J Pharmacol* 36, 170 (1969). Structure: Abraham, *Brit J Pharmacol* 36, 170 (1969). Structure: Abraham, Newton, *Biochem J* 79, 377 (1961). Nadeljak, Abraham, *Ibid* 393. In cephalosporin C_1 the amino group is replaced by a pyridinium group: Hale *et al*, *Ibid* 403. Transformation to pyridinium C_1 (as with penicillin N): Abraham *et al*, U.S. pat. 3,046,581 (1962 to Nat Res Dev Corp.). Total synthesis: Woodward *et al*, *J Am Chem Soc* 88, 852 (1966). Review of cephalosporin C and related compounds: Abraham, *Quart Rev. Chem Soc* 21, 231 (1967). Abraham, Loder, in *Cephalosporins and Penicillins*, E. H. Flynn, Ed (Academic Press, New York, 1972) pp 1-26.

water (g/100 ml): 0.037 (0°), 0.065 (20°). The only aureus in the presence of HNO_3 or nitrate.

5426. Lead Formate. $C_2H_2O_4Pb$; mol wt 297.24. C 8.08%, H 0.68%, O 21.53%, Pb 69.71%. $Pb(CHO_2)_2$.
White, lustrous prisms or needles. *Poisonous!* d 4.63. Dec at 190°. Sol in 63 parts cold water, 6 parts boiling water; insol in alcohol.

5427. Lead Hexafluorosilicate. Lead fluosilicate; lead silicofluoride. F_6PbSi; mol wt 349.28. F 32.64%, Pb 59.32%, Si 8.04%. $PbSiF_6$.
Dihydrate, colorless crystals. Sol in water. *Poisonous!*
USE: In refining lead by electrolytic methods.

5428. Lead Hydroxide. Lead oxide hydrate; basic lead hydroxide. $H_2O_4Pb_3$; mol wt 687.61. H 0.29%, O 9.31%, Pb 90.40%. $3PbO.H_2O$. Prepn and structure: Oswald et al., *Helv. Chim. Acta* 54, 1389 (1961).
White powder, absorbs CO_2 from the air. *Poisonous!* d 7.41. Insol in H_2O; sol in dil acids, fixed alkali hydroxides.

5429. Lead Hypophosphite. $H_4O_4P_2Pb$; mol wt 337.18. H 1.20%, O 18.98%, P 18.37%, Pb 61.45%. $Pb(H_2PO_2)_2$.
Hygroscopic, cryst powder, dec at elevated temps. *Poisonous!* Slightly sol in cold water, more in hot water; insol in alcohol. *Keep well closed and protected from light.*

5430. Lead Iodide. I_2Pb; mol wt 461.01. I 55.06%, Pb 44.94%. PbI_2.
Bright yellow, heavy odorless powder. *Poisonous!* d 6.16. mp 402°. One gram dissolves in 1350 ml cold, 230 ml boiling water; sol in concd solns of alkali iodides, freely sol in soln of sodium thiosulfate; sol in 200 parts cold, 90 parts hot aniline; insol in alcohol or cold HCl. *Protect from light.*
USE: Bronzing, gold pencils, mosaic gold, printing, photography.

5431. Lead Lactate. $C_6H_{10}O_6Pb$; mol wt 385.34. C 18.70%, H 2.62%, O 24.91%, Pb 53.77%. $Pb(C_3H_5O_3)_2$.
White, heavy, cryst powder. *Poisonous!* Slowly absorbs CO_2 from the air, becoming partly insol. Sol in water, hot alcohol. Keep well closed.

5432. Lead Molybdate(VI). MoO_4Pb; mol wt 367.14. Mo 26.13%, O 17.43%, Pb 56.44%. $PbMoO_4$. Occurs as the mineral wulfenite.
Yellow powder. Insol in water; sol in HNO_3 or NaOH while freshly pptd.
USE: In pigments.

5433. Lead Monoxide. Lead oxide yellow; plumbous oxide; litharge; massicot; lead protoxide. OPb; mol wt 223.20. O 7.17%, Pb 92.83%. PbO. Props of high purity PbO: Kwestroo, Huizing, *J. Inorg. Nucl. Chem.* 27, 1951 (1965); Kwestroo et al., *ibid.* 29, 39 (1967). Mfg processes: Faith, Keyes & Clark's *Industrial Chemicals*, F. A. Lowenheim, M. K. Moran, Eds. (Wiley-Interscience, New York, 4th ed., 1975) pp 509-513. Toxicity study: W. R. Bradley, W. G. Fredrick, *Ind. Med.* 10, *Ind. Hyg. Sect.* 2, 15 (1941).
Exists in two forms: red to reddish-yellow, tetragonal crystals, stable at ordinary temp; yellow, orthorhombic crystals, stable above 489°. Pauson, *J. Am. Chem. Soc.* 63, 2517 (1941). *Poisonous!* At 300-450° in the air, it converts slowly into Pb_3O_4; at higher temp reverts to PbO. d 9.53. mp 888°. Insol in water, alcohol. Sol in acetic acid, dil HNO_3, in warm solns of fixed alkali hydroxides. LD_{50} i.p. in rats: 40 mg Pb/100 g (Bradley, Fredrick).
Caution: Avoid breathing dust. Wear dust mask approved by U.S. Bureau of Mines for this purpose. Wash thoroughly before eating or smoking. Keep away from food or food products.
USE: In storage batteries, glazing pottery, preparing salts of lead or base tals. Glazing pottery, glass flux for painting on porcelain and glass; lead glass; varnishes, with glycerol as metal cement; producing cloisonné; colors on brass and bronze; coloring sulfur-containing substances, e.g., hair, nails, wool, horn; patent artificial tortoise shell and horn; pigment for rubber; vessel boiled linseed oil; an assay of gold and silver ores.

Consult the Name Index before using this section.

5434. Lead Nitrate. N_2O_6Pb; mol wt 331.21. N 8.46%, O 28.98%, Pb 62.56%. $Pb(NO_3)_2$.
White or colorless translucent crystals. *Poisonous!* d 4.53. One g dissolves in 2 ml cold, 0.75 ml boiling water, in 2500 ml abs alcohol, 75 ml abs methanol; insol in concd HNO_3. The aq soln is slightly acid: pH of 20% aq soln is 3.0-4.0.
USE: Manuf matches and special explosives, mordant in dyeing and printing calico, staining horn, mother-of-pearl, oxidizer in dye industry, sensitizer in photography, process engraving.
THERAP CAT (VET): Has been used as a caustic or escharotic.

5435. Lead Oleate. Oleic acid lead salt. Approx $Pb(C_{18}H_{33}O_2)_2$.
Granular, wax-like mass. Insol in water; sol in alc, benzene, ether, oil turpentine. LD orally in guinea pigs: 6.0 g/kg, *Handbook of Toxicology* vol. 1, W. S. Spector, Ed. (Saunders, Philadelphia, 1956) pp 176-177.
USE: In varnishes; in extreme pressure lubricants.

5436. Lead Oxalate. C_2O_4Pb; mol wt 295.22. C 8.14%, O 21.68%, Pb 70.19%.
White, heavy powder. Dec at 300°. d 5.28. *Poisonous!* Insol in water; sol in dil HNO_3, fixed alkali hydroxides; sparingly sol in oxalic acid.

5437. Lead Phosphate. $O_8P_2Pb_3$; mol wt 811.51. O 15.77%, P 7.63%, Pb 76.60%. $Pb_3(PO_4)_2$.
White powder. d 6.9. mp 1014°. *Poisonous!* Insol in water, alcohol. Sol in HNO_3, fixed alkali hydroxides.
Note: This substance may reasonably be anticipated to be a carcinogen: *Seventh Annual Report on Carcinogens* (PB95-109781, 1994) p 238.
USE: Stabilizer for styrene and casein plastics, in special glasses.

5438. Lead Selenate. O_4PbSe; mol wt 350.16. O 18.28%, Pb 59.17%, Se 22.55%. $PbSeO_4$. Prepd by adding a soln of lead nitrate to sodium selenate: Lenher, Kao, *J. Am. Chem. Soc.* 47, 1521 (1925).
Orthorhombic crystals. d^{4} 6.37. Dec by heat. Sol in concd acids, insol in water.

5439. Lead Selenite. O_3PbSe; mol wt 334.16. O 14.36%, Pb 62.01%, Se 23.63%. $PbSeO_3$. Prepd by adding selenous acid or an alkali selenite to a soln of lead chloride or nitrate: Berzelius, cited in Mellor vol. X, 833 (1930).
Powder. mp ~500°. Melts to a yellow liquid. Dec at a bright-red heat giving off selenious acid. Very sparingly sol in water, difficultly dec by boiling sulfuric acid.

5440. Lead Sesquioxide. *Lead trioxide; plumbous plumbate.* O_3Pb_2; mol wt 462.40. O 10.38%, Pb 89.62%. Pb_2O_3.
Reddish-yellow powder, converted at 370° in air to Pb_3O_4, dec at ~530° to PbO. Insol in water, dec by mineral acids or H_2SO_4, with the liberation of Cl or oxygen, respectively.

5441. Lead Sodium Thiosulfate. Lead sodium hyposulfite. $Na_4O_6PbS_4$; mol wt 635.55. Na 14.47%, O 22.66%, Pb 32.60%, S 20.17%. $Na_4Pb(S_2O_3)_3$.
White, small, heavy crystals. *Poisonous!* Sparingly sol in water; more so in thiosulfate solns.
USE: Manuf matches.

5442. Lead Stearate. Stearic acid lead salt. Approx $Pb(C_{18}H_{35}O_2)_2$.
White powder, mp ~125°. *Poisonous!* Insol in water; sol in hot alcohol.
USE: In extreme pressure lubricants, as drier in varnishes.

5443. Lead Subacetate. Lead monosubacetate; basic lead acetate. $C_4H_{10}O_8Pb_3$; mol wt 807.72. C 5.95%, H 1.25%, O 15.85%, Pb 76.96%. $Pb(C_2H_3O_2)_2.2Pb(OH)_2$.
White, heavy powder. *Poisonous!* Sol in 16 parts cold, 4 parts boiling water with alkaline reaction. On exposure to air absorbs CO_2 and becomes incompletely sol. *Keep well closed.*
USE: In sugar analysis to remove coloring matters, etc., from solns before polarizing; for clarifying and decolorizing other solns of organic substances.

5444. Lead Sulfate. O_4PbS; mol wt 303.26. O 21.10%, Pb 68.32%, S 10.57%. $PbSO_4$. Occurs as the mineral anglesite, lanarkite.
White, heavy, cryst powder. *Poisonous!* d 6.2. mp 1170°. Sol in about 2225 parts water, more soluble in dil HCl or HNO_3, less in dil H_2SO_4; sol in NaOH, ammonium acetate or tartrate solns; sol in concd hydriodic acid; insol in alcohol. LD_{50} i.p. in guinea pigs: 790 mg/kg, *Handbook of Toxicology* vol. 1, W. S. Spector, Ed. (Saunders, Philadelphia, 1956) pp 178-179.
USE: Instead of white lead as pigment; with zinc in galvanic batteries; in lithography; preparing rapidly drying oil varnishes; weighting fabrics.

5445. Lead Sulfide. PbS; mol wt 239.27. Pb 86.60%, S 13.40%. Occurs as the mineral galena. Toxicity study: W. R. Bradley, W. G. Fredrick, *Ind. Med.* 10, *Ind. Hyg. Sect.* 2, 15 (1941).
Black powder. Insol in water. Sol in HNO_3, hot, dil HCl. LD_{50} i.p. in rats: 1600 mg Pb/100 g (Bradley, Fredrick).
USE: Glazing earthenware.

5446. Lead Telluride. PbTe; mol wt 334.80. Pb 61.89%, Te 38.11%. Found in nature as the mineral altaite. Prepd from lead acetate, sodium carbonate and powdered tellurium: Montignie, *Bull. Soc. Chim. France* 1947, 750. Prepn of single crystals by heating stoichiometric quantities of the elements in a graphite cup or fused quartz tube: Brebrick, *J. Electrochem. Soc.* 104, 461 (1957).
Silver-gray cubic crystals. d^{15} 8.16. mp 905°. Most of the crystal is p-type, the n-type material being present as the surface layer. Energy gap 0.27 ev. Electron mobility 2160 cm^2/volt-sec. Hole mobility 840 cm^2/volt-sec. Resistivity 0.003 ohm-cm (p-type), 0.00090 ohm-cm (n-type). Not attacked by hydrochloric, hydrofluoric, perchloric and acetic acids at their mixtures; not attacked by soln of 30% potassium hydroxide or of alkali metal sulfides. Dil nitric acid turns the surface black, while concd nitric and fuming acids gray surface and turns the black surface to gray. Hot concd sulfuric acid produces a reddish-violet surface.
USE: In photoconductive cells, in semiconductor research.

5447. Lead Tetraacetate. $C_8H_{12}O_8Pb$; mol wt 443.38. C 21.67%, H 2.73%, O 28.87%, Pb 46.73%. $Pb(CH_3COO)_4$. Prepd from Pb_3O_4 and glacial acetic acid preferably in the presence of some acetic anhydride: Dimroth, Schweizer, *Ber.* 56, 1375 (1923); Bader, *Inorg. Syn.* 1, 47 (1939); Brauer in *Handbook of Preparative Inorganic Chemistry* vol. 1, G. Brauer, Ed. (Academic Press, New York, 2nd ed., 1963) p 767. Prepn by electrolysis: Prokhorov, Oxakov, *Dokl. Akad. Nauk SSSR* 112, 307 (1957), *C.A.* 51, 16146 (1957). Samoilov et al., *Khim. Prom. (Moscow)* 46, 897 (1970), *C.A.* 74, 49800 (1971). Reviews of prepn and use as oxidizing agent: Criegee, "Oxidations with Lead Tetraacetate" in *Oxidation in Organic Chemistry*, Part A, K. B. Wiberg, Ed. (Academic Press, New York, 1965) pp 277-366; Zyka, *Pure Appl. Chem.* 13, 569-581 (1966).
Colorless monoclinic prisms from glacial acetic acid. Turns pink readily. Unstable in air. Hydrolyzed by water with the formation of brown lead dioxide and acetic acid. Faint odor of vinegar. d^{17} 2.228. mp 175-180°. Sol in hot glacial acetic acid, benzene, chloroform, tetrachloroethane, nitrobenzene. Dissolves in concd halogen acids with the formation of haloplumbic acids, H_2PbX_6. The dry material can be stored in sealed, evacuated ampuls.
USE: Selective oxidizing agent in organic syntheses: Criegee, *Angew. Chem.* 53, 321 (1940); *Newer Methods of Preparative Organic Chemistry* (Interscience, N.Y., 1948) pp 1-20.

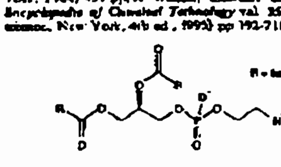
5448. Lead Tetrafluoride. Plumbic fluoride. F_4Pb; mol wt 283.19. F 26.83%, Pb 73.17%. PbF_4. Prepd by passing fluorine diluted with CO_2 or N_2 over PbF_2 at 300°: v. Wartenberg, *Z. Anorg. Allgem. Chem.* 244, 339 (1940). Review: Kemmitt, Sharp, *Advan. Fluorine Chem.* 4, 167 (1965).
White, tetragonal crystals. d 6.7. mp ~600°. Readily hydrolyzes and forms brown flakes (PbO_2) in the presence of moisture.
USE: Has been proposed as a fluorinating agent for hydrocarbons.

5449. Lead Tetroxide. Lead oxide red; red lead; minium; lead orthoplumbate; mineral orange; mineral red; Paris red; Saturn red; C.I. Pigment Red 105; C.I. 77578. O_4Pb_3; mol wt 685.60. O 9.33%, Pb 90.67%. Pb_3O_4. The article of commerce contains about 90% Pb_3O_4; the remainder being chiefly lead monoxide. Prepn: M. Baudler in *Handbook of Preparative Inorganic Chemistry* vol. 1, G. Brauer, Ed. (Academic Press, New York, 1963) pp 755-757; Straumanis, S. T. Orme, *J. Am. Chem. Soc.* 65, 1107 (1943); *Aroma Mellor*, vol 7 (1930) pp 672-680.
Bright-red, heavy powder. *Poisonous!* Dec at ~500° with evolution of oxygen. d 9.1. Insol in water or alcohol; sol in excess glacial acetic acid, in hot HCl with evolution of Cl, in dil HNO_3 in presence of H_2O_2. LD_{50} i.p. in guinea pigs: 220 mg/kg, *Handbook of Toxicology* vol. 1, W. S. Spector, Ed. (Saunders, Philadelphia, 1956) pp 176-177.
USE: Plasters and cements, manuf colorless glass, glass for furnaces, flux for porcelain painting, protective paint for iron and steel, oil-color for ship paints, varnishes; coloring rubber; cement for glass, gas and steam pipes, storage batteries, pencils for writing on glass, manuf lead peroxide, matches.

5450. Lead Thiocyanate. Lead sulfocyanate. $C_2N_2PbS_2$; mol wt 323.37. C 7.43%, N 8.66%, Pb 64.07%, S 19.83%. $Pb(SCN)_2$. Prepn: Goodrich, Weinberger, *Inorg. Syn.* 1, 85 (1939).
White, odorless powder. d 3.82. *Poisonous!* Sol in ~200 parts cold, 50 parts boiling water, also sol in alkali hydroxide and thiocyanate solns.
USE: Reserve dyeing with aniline black; manufacture of safety match heads and cartridges.

5451. Lead Vanadate(V). Lead metavanadate. O_6PbV_2; mol wt 423.08. O 22.70%, Pb 51.15%, V 24.15%. $Pb(VO_3)_2$.
Yellow powder. Insol in water. Dec by HNO_3.
USE: Manuf other vanadium compds; as pigment.

5452. Lecithin. Phosphatidylcholine; Lecithol; Vitellin; Kelecin; Granulestin. Phospholipid found in all living organisms (plants and animals). Significant constituent of nervous tissue and brain substance. A mixture of the diglycerides of stearic, palmitic, and oleic acids, linked to the choline ester of phosphoric acid. Commercial grades contain 2.2% P. Isoln from eggs: Sinclair, *Can. J. Res.* 26B, 777 (1948). Product of commerce is predominantly soybean lecithin obtained as a by-product in the manuf of soybean oil: Stanley in K. S. Markley, *Soybeans* vol. 2 (Interscience, New York, 1951) pp 593-647. Soybean lecithin contains palmitic acid 11.7%, stearic 4.0%, palmitoleic 8.6%, oleic 9.8%, linoleic 55.0%, linolenic 4.0%, C_{20} to C_{22} acids (includes arachidonic) 5.5%. Synthesis of a mixed acid α-lecithin: de Haas, van Deenen, *Tetrahedron Letters* 1960 (no. 9), 1. Synthetic L-α-(distearoyl)lecithin is identical with hydrogenated egg yolk lecithin and L-α-(dipalmitoyl)lecithin is identical with ... palmitate, q.v., a natural phosphatide of brain, lung, and spleen. Commercial grades of natural lecithin are reported to contain a potent vasodepressor substance: McQuarrie, Anderson, U.S. pat. 2,945,869 (1960 to Cutter Labs.). Comprehensive monograph: G. B. Ansell, J. N. Hawthorne, *Phospholipids* (Elsevier, New York, 1964) 439 pp. A. Wendel, "Lecithin" in Kirk-Othmer *Encyclopedia of Chemical Technology* vol. 15 (Wiley-Interscience, New York, 4th ed., 1995) pp 192-210.



Waxy mass when the acid value is about 20. Pourable, thick fluid when the acid value is around 30. Color is nearly white when freshly made, but rapidly becomes yellow to brown in air. d^{24} 1.0305. Iodine value 95, saponification

Consult the Name Index before using this section.

water 1lb. Insoluble but swells up in water and in polar solv forming a colloidal suspension. Soluble in about 12 parts acid, also alcohol, and in chloroform, ether, petr ether, in ethanol cells and fatty acids, sparingly sol in benzene. Based preparations practically toxic to cold vegetable and animal cells.

Use Edible and digestible antidotal and emulsifier of natural origin. Used as margarine, chocolate and ice (ice cream) to prevent ice crystal formation and recrystallization of other industrial uses, e.g. treating leather and textiles

THERAP CAT Lipotropic

3451. Lectins. *Agglutinins; adhesins; phytoagglutinins, phasins, proteotoxic.* A group of proteins, widely distributed in nature. Distinguished by ability to agglutinate erythrocytes and many other types of cells. Although these substances have been known since 1888, when Stillmark isolated a hemagglutinin from castor beans, the term "lectin" (from the Latin legere, to choose) was first introduced by W. C. Boyd and E. Shapleigh in Science 119, 419 (1954). It is now used to designate "a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates." J. Goldstein *et al.*, Nature 285, 66 (1980). Lectins are found primarily in seeds of plants, but also roots in roots, leaves and bark. In addition, they are present in invertebrates such as clams, snails, and horseshoe crabs, and in several vertebrate species. The term phytohemagglutinin is used to refer to plant lectins. Important members of the lectin family include concanavalin A, abrin, ricin, crotin, soybean agglutinin or SBA. Lectins may considerably in chemical and physical properties, only a limited number have been purified. Mild uts of 17,000 to 400,000 have been reported and metal lectins have been found to contain Mn^{++} and Ca^{++}. Nearly all lectins seem to be activated by free sugars or monosaccharides of appropriate specificity. Although their physiological functions in plants or in other organisms are unknown, lectins exhibit a variety of unusual biological properties. Some are specific in their reactions with human blood groups; some induce mitosis in lymphocytes. WGA from wheat germ lipids has been shown to agglutinate mouse tumor cells more readily than cells from normal tissue. I. C. Aub *et al.*, Proc. Nat. Acad. Sci. USA 60, 643 (1968); M. M. Burger, A. R. Goldberg, ibid 57, 359 (1967). Soybean agglutinin and concanavalin A have been shown to agglutinate cell lines transformed by virus or chemical carcinogens: M. Inbar, L. Sachs, Nature 223, 710 (1969); Proc. Nat. Acad. Sci. USA 63, 1418 (1969); B. A. Sela *et al.*, J. Membrane Biol. 3, 267 (1970). Some plant lectins cause the direct release of insulin or another envelope phosphorylase: F. Portella *et al.*, Science 201, 467 (1963). Soybean agglutinin has also been used to favor maternal transplants in patients with severe combined immunodeficiency: Y. Reisner *et al.*, Blood 61, 341 (1983). Reviews: N. Sharon, H. Lis, Science 177, 949-959 (1972); eidem, Ann. Rev. Biochem. 42, 541-574 (1973); L. Sequeira, Ann. Rev. Phytopathol. 16, 453-481 (1978); Book Lectin Biology; Biochemistry; Clinical Biochemistry vol. 1, T. C. Boyd-Harrison, Ed. (in Organic, New York, 1981) 414 pp.

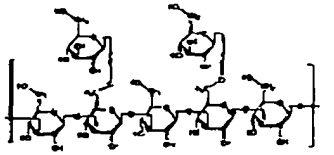
USE: As tools for studying cell surface properties, in cancer research.

3452. Ledol. *(1aR-(1aα,4aβ,7α,7aβ,7bα))-Decahydro-1,1,7-trimethyl-1H-cycloprop[e]azulen-4-ol*, "Ledum camphor"; $C_{15}H_{26}O$, mol wt 222.37. C 81.02%, H 11.79%, O 7.19%. Occurs in the essential oil from leaves of *Ledum palustre* L.: Grassmann, *Ropen Pharm.* 38, 53 (1935); Hjelt, *Ber.* 28, 3467 (1895), from *L. groenlandicum* Oeder, *L. palustrum* Piper, *Brassicus*. Colin Lyon, *J. Am. Pharm. Assoc. Sci. Ed.* 39, 306 (1950). Structure: O. Bilde *et al.*, *Tetrahedron Letters* 1959 (no 6), 14. Stereochemistry: L. Dolejs, F. Sorm, *Tetrahedron Letters* 1959 (no 17), 1. Ir and nmr spectrochemistry: O. Bilde *et al.*, *J. Am. Chem. Soc.* 83, 4472 (1960).

Needles from alc, mp 104-105°. Sublimes easily, even below the mp. $[\alpha]_{589}$ 7.5°, n_D^{21} 1.4667. (+)-form: +79° (c = 10 in chloroform). Practically insol in water. Sol in alc (about 10% w/v). Soluble in other organic solvents.

Chromic $C_{30}H_{42}O_3$, ruby-red prisms, mp 97°. $[\alpha]_D$ +30° (c = 3 in chloroform).

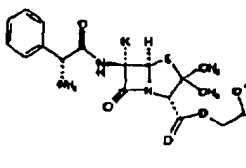
3453. Lentinan. *(β)-1,6(1,3-Diran)-3-glucose*, schizophyllan; (→3)-β-D-Glcp-(1→6)-β-D-Glcp-(1→ repeating unit with every third glucose unit linked to the next unit by a single β-1,6 linkage; $(C_6H_{10}O_5)_n$, mol wt 300,000-1,000,000. Neutral polysaccharide isolated from the edible mushroom *Lentinus edodes* (Berk.) Sing. Primary structure is a β-1,3-D-glucan having 2 β-1,6-glucopyranoside branchings for every 5 β-1,3 linkages. Isolation and antitumor activity: G. Chihara *et al.*, *Nature* 222, 687 (1969); *Cancer Chemother. Rep.* 59, 2735 (1975); immunostimulant activity: Y. Y. Maeda, G. Chihara, *Nature* 229, 634 (1971). Preparation of stable aqueous solutions: M. Fuji *et al.*, Neth. pat. Appl. 7,601,916 corresponding to U.S. pat. 4,101,532 (1976, 1978 both to Ajinomoto). Monograph. Structural study: T. Sasaki *et al.*, *Carbohydrate Res.* 47, 99 (1976). Preclinical evaluation and toxicity studies: G. Chihara, *Adv. Exp. Med. Biol.* 166, 189 (1983). Mechanism of action studies: H. Miyahashi, T. Aoki, *Int. J. Immunopharmacol.* 4, 365, 373 (1982). Clinical trial in gastric and colorectal cancer: T. Taguchi *et al.*, *Adv. Exp. Med. Biol.* 166, 161 (1983); K. Okamura *et al.*, *Cancer* 55, 2498 (1985). Review: G. Chihara, *Int. J. Tissue Res.* 4, 207-225 (1982).



White powder, dec 240°. Sol in aqueous alkali, formic and trichloroacetic acid, hot water. DMSO. Insol in cold water, alcohol, ether, chloroform, pyridine, hexamethylphosphoramide. Subtle against adherent and hydrophobic cells [α]$_D^{20}$ +13.3-14.5° (in 3% NaOH). ϵ 19,531.5 (in 10% NaOH).

THERAP CAT Antineoplastic, immunomodulator.

3454. Leflunomide. *4-Isoxazolecarboxamide, 5-methyl-N-(4-(trifluoromethyl)phenyl)-*; $C_{12}H_9F_3N_2O_2$, mol wt 270.21. C 53.34%, H 3.36%, F 21.09%, N 10.37%, O 11.84%. Prepn: F. Kohno *et al.*, Eur. pat. Appl. 326,406, 326,407 (1989, both to Hoechst-Roussel); F. Kohno *et al.*, *Chem. Pharm. Bull.* 38, 2611 (1990). Immunosuppressive activity: M. Kaltenhauser *et al.*, *Arzneim. Forsch.* 41, 1001 (1991). Pharmacokinetics: M. Kaltenhauser *et al.*, *Int. J. Clin. Pharmacol. Ther. Toxicol.* 29, 341 (1991). Clinical trial in rheumatoid arthritis: P. Emery *et al.*, *Arthritis Rheum.* 34, 1394 (1991). Review: R. Fox, *Clin. Immunol. Newsletter* 12, 1 (1992).

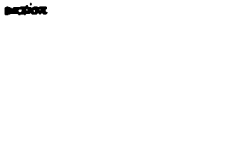


Crystals from methanol or dioxane, mp > 300° (Sasaki, Fujimura). Also reported as light grey crystals from DMF, mp 310°-312° (Sakanoue, Ishii). Technical product properties: odorless, white to tan crystalline solid, d^{20} 1.33 g/cc. Vapor pressure (25°) 0.3 mPa. Sol in water 6 mg/l, acetone 2g/l. LD_{50} (80% wettable powder) orally in rats > 10,000 mg/kg; LC_{50} 96 hr in bluegill sunfish: unaffected (50-1000, 125 mg/l). LC_{50} 8 day in bobwhite quail, Peking duck (mg/kg): 2000, > 5430 (DuPont technical data sheet)

USE: Herbicide

3455. Leghemoglobin. *Leguminous hemoglobin.* A red pigment present in the root nodules of leguminous plants. Isolated from soya beans: Keilin, Wang, *Nature* 155, 227 (1945); Appleby, *Biochim. Biophys. Acta* 60, 28 (1962). Heme is in approx one-fourth that of hemoglobin: Ehrenberg, Elfstedt, *Acta Chem. Scand.* 17, 2343 (1963). Released into four components on DEAE-cellulose column: Bilfeldt, *ibid* 24, 609 (1969). Suggested to act as an oxidation catalyst in the symbiotic nitrogen fixation: *Ann. Bot.* 25, 913 (1961). Primary structure of soybean leghemoglobin: Efstedt, *Biochem. Biophys. Res. Commun.* 77, 1511 (1977); *Eur. J. Biochem.* 94, 39, 53 (1979).

3456. Leiopyrrole. *N,N'-Diethyl-3-(2-(2-methyl-3-phenyl-1-pyrrolinyl)ethanamine; 1-(o-Ethoxyphenoxy)-3-methyl-3-phenylpyrrole; 3-methyl-1-(2-(o-ethylphenoxy)ethoxyphenyl)-3-phenylpyrrole*, DY-714. $C_{21}H_{26}N_2O$, mol wt 322.49. C 78.23%, H 8.13%, N 8.69%, O 4.96%. Preparation: Suzuki *et al.*, *J. Med. Pharm. Chem.* 1, 23 (1959).



Base, $bp_{0.5}$ 252°, n_D^{20} 1.6025

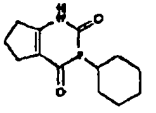
3457. Leiopyrrole. *N,N'-Diethyl-3-(2-(2-methyl-3-phenyl-1-pyrrolinyl)ethanamine; 1-(o-Ethoxyphenoxy)-3-methyl-3-phenylpyrrole; 3-methyl-1-(2-(o-ethylphenoxy)ethoxyphenyl)-3-phenylpyrrole*, DY-714. $C_{21}H_{26}N_2O$, mol wt 322.49. C 78.23%, H 8.13%, N 8.69%, O 4.96%. Preparation: Suzuki *et al.*, *J. Med. Pharm. Chem.* 1, 23 (1959).

Base, $bp_{0.5}$ 252°, n_D^{20} 1.6025

3458. Lemon Peel. *Citrus rind* or fresh ripe fruit of *Citrus limonum* (L.) Risso (C. medica var. limon L.), Rutaceae. Habit: Northern India, cultivated in California, West Indies, Italy, Spain, Greece. Volatile oil, lemonade, bitter principle.

USE: As a flavor in medicines, also in beverages, confectionery, and cooking.

3459. Lenacil. *3-Cyclohexyl-6,7-dihydro-1H-cyclopentapyrimidine-2,4(3H,5H)-dione, 3-cyclohexyl-5,6-trimethylene uracil, 3-cyclohexyl-1,5,6,7-tetrahydro-2H-cyclopentapyrimidine-2,4(3H)-dione*, Du Pont 634, Venzar. $C_{13}H_{18}N_2O_2$, mol wt 234.30. C 66.64%, H 7.74%, N 11.96%, O 13.66%. Preps: S. Smolin, H. Fujimoto, Japan. pat. 63 08787 (1963). *CA* 59, 642g (1963), and herbicidal activity: B. J. Lehmkuhl, U.S. pat. 3,235,360 (1966 to Du Pont, Monsanto). Synthesis: S. Senda *et al.*, *Chem. Pharm. Bull.* 21, 1904 (1973). Mode of action: C. E. Hoffmann, *Pestic. Chem., Proc. Int. Pest Control Congr. Pestic. Chem.*, 3, 4, 5 Tokyo, Ed., (Geraldo and Kenneth Kearney Publishers, New York, 1972) pp 65-95. Field evaluations: J. G. Wilson, W. E. Bray, *Proc. 12th Brit. Weed Control Conf.* 485 (1974). GC determination in soils: D. J. Cassidy, R. C. Dunn, *J. Agric. Food Chem.* 25, 376 (1977). Soil persistence: M. Tomo *et al.*, *Weed Res.* 23, 245 (1983). Field tolerance: D. V. Clay, *Proc. Aberdeen-Colloq. Pestic.* 10 (1980).



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USE: Herbicide

3460. Lenampicillin. *(2S-(2α,5α,6β(S*))-6-(((Aminophenylacetyl)amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester, ampicillin (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl ester, 6-[D(-)-α-aminophenylacetamido]penicillanic acid 15-methyl-2-oxo-1,3-dioxol-4-ylmethyl ester*; $C_{25}H_{27}N_3O_8S$, mol wt 561.57. C 56.60%, H 5.07%, N 7.78%, O 24.27%, S 5.95%. Orally active ampicillin prodrug. Preps: F. Ishimoto *et al.*, Eur. pat. Appl. 39,044, also, U.S. pat. 4,342,693 (1982, 1983 both to Kaneka); F. Ishimoto *et al.*, *Chem. Pharm. Bull.* 33, 3241 (1984). Esterase levels in rat, dog, and human blood: Ishimoto *et al.*, *ibid.* 4316. Metabolism in man, dogs, rats: Yamada *et al.*, *Japan J. Antibiot.* 38, 1716, 1765; *CA* 104, 161478a, 225508r (1986). Human pharmacokinetics: A. Kase, M. Takashima, *Antimicrob. Ag. Chemother.* 29, 941 (1986). Review of studies on antibacterial activity, mechanism, pharmacology, clinical trials: *Chemotherapy* (Tokyo) 34, Suppl. 1, 1-1000 (1986).

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USE: Herbicide

3461. Lenampicillin. *(2S-(2α,5α,6β(S*))-6-(((Aminophenylacetyl)amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester, ampicillin (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl ester, 6-[D(-)-α-aminophenylacetamido]penicillanic acid 15-methyl-2-oxo-1,3-dioxol-4-ylmethyl ester*; $C_{25}H_{27}N_3O_8S$, mol wt 561.57. C 56.60%, H 5.07%, N 7.78%, O 24.27%, S 5.95%. Orally active ampicillin prodrug. Preps: F. Ishimoto *et al.*, Eur. pat. Appl. 39,044, also, U.S. pat. 4,342,693 (1982, 1983 both to Kaneka); F. Ishimoto *et al.*, *Chem. Pharm. Bull.* 33, 3241 (1984). Esterase levels in rat, dog, and human blood: Ishimoto *et al.*, *ibid.* 4316. Metabolism in man, dogs, rats: Yamada *et al.*, *Japan J. Antibiot.* 38, 1716, 1765; *CA* 104, 161478a, 225508r (1986). Human pharmacokinetics: A. Kase, M. Takashima, *Antimicrob. Ag. Chemother.* 29, 941 (1986). Review of studies on antibacterial activity, mechanism, pharmacology, clinical trials: *Chemotherapy* (Tokyo) 34, Suppl. 1, 1-1000 (1986).

Crystals from methanol or dioxane, mp > 300° (Senda, Fujimura). Also reported as light grey crystals from DMF, mp 310°-312° (Sakanoue, Ishii). Technical product properties: odorless, white to tan crystalline solid, d^{20} 1.33 g/cc. Vapor pressure (25°) 0.3 mPa. Sol in water 6 mg/l, acetone 2g/l. LD_{50} (80% wettable powder) orally in rats > 10,000 mg/kg; LC_{50} 96 hr in bluegill sunfish: unaffected (50-1000, 125 mg/l). LC_{50} 8 day in bobwhite quail, Peking duck (mg/kg): 2000, > 5430 (DuPont technical data sheet)

USE: Herbicide

3462. Lenercin. *4-O-(6-Deoxy-2,3-dimethoxybenzoyl-α-D-lincosaminide 6-(methylthiocarbonyl)-4-hydroxy-L-proline ester; 4-hydroxy-L-proline ester of 6-deoxy-6-(1-methyl-4-propyl-2-pyrrolidinecarboxamido)-1-thio-α-D-erythro-α-D-galacto-octopyranoside 2,3-dimethoxybenzoate*; $C_{31}H_{51}N_3O_9S_2$, mol wt 673.90. C 55.25%, H 7.63%, N 6.24%, O 21.37%, S 9.51%. Prepn: Kubota *et al.*, *Yakugaku Zasshi* 87, 1547 (1967); *CA* 68, 59744 (1968). Structure: Kubota *et al.*, *Yakugaku Zasshi* 87, 1553 (1967); *CA* 68, 59745 (1968). Antibacterial activity: Kubota *et al.*, *Yakugaku Zasshi* 87, 1559 (1967); *CA* 68, 59746 (1968). Pharmacology: Kubota *et al.*, *Yakugaku Zasshi* 87, 1564 (1967); *CA* 68, 59747 (1968). Clinical trial: Kubota *et al.*, *Yakugaku Zasshi* 87, 1570 (1967); *CA* 68, 59748 (1968). Review: Kubota, *Antibiotics Chemother.* 16, 1-11 (1971).

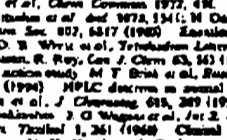
and toxicology. Bayer et al. *J Pharmacol Exp Ther* 121, 210 (1957)
Liquid. bp$_{5}$ 185-195°.

Hydrochloride. $C_{16}H_{35}NO_3 \cdot HCl$, crystals from methanol + ether, mp 124-127°.
THERAP CAT Antisperm acidic

6890. Spermaceti. Mixt. Dried leaves and tops of *Mentha spicata* L. (*M. viridis* L.), *Lamiaceae*. Arab. *Asas*. Europe. Asia widely cultivated in U.S. Principal active constit. is a volatile oil which contains at least 50% carvone.
USE As flavor.

6891. Spermidine. Mixt. Dried leaves and tops of *Mentha spicata* L. (*M. viridis* L.), *Lamiaceae*. Arab. *Asas*. Europe. Asia widely cultivated in U.S. Principal active constit. is a volatile oil which contains at least 50% carvone.
USE As flavor.

6892. Spermidine. *1H-12a,4a5-3a8,6a7,8a9,8a11,10a12-Decahydro-4a,7,9-trihydroxy-3-methyl-6,8-bis(methylamino)-4H-pyrano[2,3-b]-[1,4]benzodioxin-4-one*; an aminoglycoside antibiotic; crystalline; CAS-1100; M 481. $C_{21}H_{43}N_5O_7$; anol wt 333.35. C 50.59%, H 7.28%, N 6.43%, O 33.70%. Antibiotic isolated from *Streptomyces griseoluteus* n. sp. Waksman et al. *J Bacteriol* 40, 581 (1940); Waksman & Lechevalier, 11, 146 (1961); M. E. Bunge et al. *Ibid.* 64, 1; M. Bunge, C. De Boer, U.S. pat 3,334,082 (1966 to Upjohn). Purification and crystallization: H. K. Jahnke, U.S. pat 3,386,960; V. J. Peters, U.S. pat 3,772,266 (1965, 1966 both to Upjohn). Isolation and characterization: A. C. Ritchie, A. F. Wagner, *Antibiotics Ann.* 1956/57, 503. Structure: H. Hachmann et al. *J Am Chem Soc* 84, 3113 (1962); P. F. Wiley et al. *Ibid* 84, 3113 (1962). Solubility data: J. R. Mandl, P. J. Wise, *J Assoc. Offic Anal Chem* 50, 437 (1967). Biochemistry and mechanism of action: T. G. Cochran et al. *Chem Commun* 1972, 491. Biosynthesis: L. A. Mitscher et al. *Ibid* 1973, 1341; M. Daniels et al. *J Am Chem Soc* 107, 6817 (1985). Enzyme-inducing systems: D. B. White et al. *Tetrahedron Letters* 1979, 2157; J. Hannaman, R. Frey, *Can J. Chem* 63, 145 (1985). Mechanism of action study: M. F. Brink et al. *Biochim Biophys Acta* 823, 325 (1990). HPLC detection on several phases: N. Haagsma et al. *J Chromatog* 618, 269 (1993). Clinical pharmacokinetics: J. G. Wagner et al. *Int J. Clin Pharmacol Ther Toxicol* 1, 261 (1968). Clinical evaluation in pneumonia: Y. H. Kami et al. *Geriatrics Ibid* 45, 140 (1990); in ophthalmol: M. Ostrow et al. *Int. Ophthalmol Clin* 29, 291 (1989). Occupational exposure: C. M. Venning et al. *Ann Occup Hyg* 37, 256 (1993). Review: W. M. McCormack, M. Finland, *Ann Intern Med* 64, 712-716 (1966); W. J. Holloway, *Med Clin North Am* 56, 1169-1173 (1972).



Amorphous powder. $[\alpha]_D^{25}$ +23° (water). pKa', 6.95, pKa'', 8.90. Sol in water, methanol, ethanol. Insol in acetone, hydrocarbon solvents.

Dihydrochloride, pentahydrate. $C_{21}H_{43}N_5O_7 \cdot 2HCl \cdot 5H_2O$. *Apamycin, Spectogard, Linolin, Tapamycin, Tavomix, Trefimix.* Exists as a bluish hydrate and not as the anhydrous form (Cochran et al.). Colorless needles from aq acetone, mp 201-207° (dec). $[\alpha]_D$ +24.8° (c = 0.42 in water). Sol in water, methanol, propylene glycol, formamide. DMSO, 0.1N NaOH, 0.1N HCl: >20 mg/ml. Sol (mg/ml) chloroform 0.062, acetone 0.015, diethyl ether 0.010.

Sulfate tetrahydrate. $C_{21}H_{43}N_5O_7 \cdot H_2SO_4 \cdot 4H_2O$, white crystals from aq acetone, mp ~185° (dec). $[\alpha]_D^{25}$ +77.8° (c = 1 in water). pKa', 7.00, pKa'', 8.73. Sol in water, DMSO 21.3, 5-10 mg/ml. Practically insol in pyridine, chloroform, ethanol, ethyl acetate, cyclohexane, benzene, acetone, DMF, dioxane, acetonitrile.
THERAP CAT Antibacterial(s)
THERAP CAT DICT Antibacterial(s)

6893. Spectrin. Taken A. Major protein component of the membranous cytoskeleton of mammalian and avian erythroid cells. Responsible for maintaining the normal shape, strength and stability of the erythrocyte. A ubiquitous family of proteins; α-actinin analogs have also been isolated from a variety of non-erythroid cells. Given the exact system because of its original isolation from hemoglobin and cell membranes known as erythrocyte "ghosts". V. F. Marchesi, E. Steers, *Science* 159, 203 (1968). Comparison with actin, q.v.: E. Steers, V. Marchesi, *J Gen Physiol* 61, 65 S (1969). Cells from human red cells and various comparison: T. W. Tillack et al., *Biochim Biophys Acta* 300, 17 (1973). Spectrin is a heterodimer composed of 2 high mol wt polypeptide subunits referred to as band 1 or α-spectrin (mol wt ~240 kDa) and band 2 or β-spectrin (mol wt ~220 kDa). Structural studies: M. Clarke, *Biochim Biophys Res Commun* 65, 1061 (1975); T. L. Stack, *J Cell Biol* 67, 1 (1974); J. M. Anderson, *J Biol Chem* 254, 1970 (1979); D. W. Speicher et al., *Ibid* 255, 9087 (1980). The subject of spectrin is traced to the red cell membrane by process known as analysis: V. Bennett, P. J. Stenbuck, *Ibid* 254, 2533 (1979), or synthesis: J. Yu, S. R. Goodman, *Proc Nat Acad Sci USA* 76, 2340 (1979). The spectrin heterodimers aggregate on the membrane surface and, in contact with actin and other proteins, form a filamentous network that covers the surface of the cytoplasmic membrane: S. E. Lux, *Nature* 265, 420 (1979); idem, *Ann Hematol* 56, 21 (1979). Identification of spectrin analogs from nonerythroid cells: S. R. Goodman et al., *Proc. Nat Acad Sci USA* 78, 7570 (1981). Cells of fetrin, also known as brain or neural cell spectrin: J. Levine, M. Willard, *J Cell Biol* 90, 631 (1981); V. Bennett et al., *Nature* 299, 126 (1982). Isolation of TNP 260/240, a spectrin analog, from chicken intestinal epithelial cells: J. R. Glenney et al., *Cell* 24, 845 (1981). Comparison of erythroid and nonerythroid spectrin: J. R. Glenney, P. Glenney, *Eur J Biochem* 146, 539 (1984). Review of erythrocyte spectrin: S. R. Goodman, K. Bullo, *Am J Physiol* 244, C121-C131 (1983), of several cell spectrins: S. R. Goodman, I. S. Zagon, *Ibid* 284, C347-C360 (1983). Cytoskeletal review: S. R. Goodman et al., *Crit Rev Biochem* 23, 171-234 (1988).

6894. Spermaceti, Crude mixt. Spermwax. A wax substance from the head of the sperm whale, *Cetus*. Chiefly cetyl palmitate, but cetyl alcohol present in appreciable amounts; esters of lauric, stearic, and myristic acids, esters of higher alcohols also present.

White, somewhat translucent, slightly unctuous mass with crystalline fracture and pearly luster, almost odorless and tasteless but becomes yellow and rancid on long exposure to air. d 0.935-0.944, mp 42-50°, eff ~14.330. Saponification value 120-136. Iodine no. 3-14. Used in waxes or cold cream. Sol in chloroform, ether, carbon disulfide, oils, boiling alcohol, slightly sol in cold ether.

USE As a base for ointments, creams, etc., and in candles with egg yolk as expressed almond oil. In manufacture of candles, soaps, cosmetics, laundry wax, finishing and tanning. Lower Emollient

6895. Spermidine. *N-[3-Aminopropyl]-1,4-butanediamine*, *N-(3-aminopropyl)tetramethylenediamine*. $C_7H_{19}N_3$; mol wt 145.25. C 57.88%, H 13.18%, N 28.93%. $NH_2(CH_2)_3NH(CH_2)_4NH_2$. Biogenic polyamine formed from putrescine, a precursor of spermine, q.v. First detected in human sperm, but occurs widely in nature. Essential for both normal and neoplastic tissue growth. Synthesis: H. W. Dudley et al., *Biochem J* 28, 1063 (1934), 31, 971 (1937); F. V. Brown, W. Vaidyanathan, *Ber* 70, 1234 (1937). M. Bergmann, H. F. Schlebe, *J Am Chem Soc* 74, 1836 (1952). For reviews of the early literature, see N. Campbell, *Die Enzymatische Arbeit* [5 Kongr., Basel, 4th ed., 1936] 619 pp; H. Tabor et al., *Ann Rev Biochem* 30, 579-604 (1961). Role on cell growth process: C. W. Tabor, H. Tabor, *Ibid* 45, 285 (1976); *J. Invest. et al. Biochem Biophys Acta* 473, 341 (1978); C. W. Porter, R. J. Bergeron, *Science* 219, 661 (1983). Formation and interconversion of spermidine and putrescine in mammalian cells: A. E. Pegg et al. *Adv Enzyme Regul* 18, 427 (1980). Effect on polycystic ovary disease in vivo: A. K. Abraham, A. Pitt, *Eur J Biochem* 186, 333 (1990). Regulation of rRNA methylase transcription: M. Mach et al., *Biochem J* 287, 153 (1992). HPLC study: C. E. Frumel, D. M. Russell, *J. Chromatog*

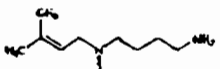
Consult the Name Index before using this section.

378, 61 (1987). Interactions with actin, q.v.: C. Orrell-Jones, *Biochem Biophys Res Commun* 186, 1096 (1992). Studies on use as a biochemical tool in cancer research: A. Thyss et al. *J Cancer Clin Oncol* 19, 611 (1982); V. Gennaro et al. *J Nat Prod.* 45, 608 (1982); C. W. Porter et al., *J Nat Prod.* 45, 4072 (1983). Toxicity study: P. R. Lang, *Cancer Res* 42, 4072 (1983). Toxicity study: P. R. Lang, Toxicol et al. *J Antibiot* 36, 1361 (1983). Review: T. C. Thatcher, *Life Sci* 32, 787-793 (1983); D. Hicks, *Differentiation* 19, 1-20 (1981); L. Sherman, *Med Biol* 60, 106-113 (1982). Basic *Polyamines in Biology and Medicine*, D. R. Morris, L. J. Marton, Eds (Dekker, New York, 1981) 517 pp.
mp 126-130°. Sol in water, ethanol, ether.
Tetrahydrochloride. $C_{11}H_{30}Cl_4N_4$, crystal from ethanol/ether, mp 256-258°.
USE As a tool in biochemical research.

6896. Spermine. *N,N'-Bis(3-aminopropyl)-1,4-butanediamine*, *N,N'-bis(3-aminopropyl)butane-1,4-diamine*, *spermine*, *tetraaminoamine*, *norspermine*. $C_{10}H_{26}N_4$; mol wt 202.34. C 59.36%, H 12.95%, N 27.69%. $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. Biogenic polyamine formed from spermidine, q.v., and occurring in almost all tissues. Essential for both normal and neoplastic tissue growth. First observed in human semen and described as the crystalline phosphate salt by A. von Leeuwenhoek, *Phil Trans Roy Soc London* 32, 1678 (1674). For a description of the historic discovery, isolation, and early properties of spermine, see *Bolzmin* vol. IV, Sappi 1, 784; 84 Congressione, *Die Biogenen Amine* (S. Karger, Basel, 4th ed., 1951) 619 pp; H. Tabor et al., *Ann Rev. Biochem* 30, 579-604 (1961). Paper of spermine and its tetrahydrochloride: Brand et al., *J Biol Chem* 73, 790 (1946). Role in cell growth processes: C. W. Tabor, H. Tabor, *Ann Rev Biochem* 45, 285 (1976); J. Janne, *Biochim Biophys Acta* 473, 241 (1978). Modulator of calcium-dependent exocytosis processes: T. C. Theoharides, *Life Sci* 33, 703 (1983). Biosynthesis in tissue: L. Sherman, *Med Biol* 60, 106 (1961). HPLC study: C. E. Frumel, D. M. Russell, *J Chromatog* 339, 47 (1985). Use as a biochemical marker for malignant tumors: V. Heiss et al., *Cancer Res* 43, 3348 (1983). Metabolic study: A. E. Pegg et al., *Biochemistry* 21, 3063 (1982). Review of role in cell proliferation and differentiation: D. Hicks, *Differentiation* 19, 1-20 (1981). Book *Polyamines in Biology and Medicine*, D. R. Morris, L. J. Marton, Eds (Dekker, New York, 1981) 512 pp.
Needles, mp 35-40°. Liq, bp_{760} 141-142°. Strong base. Absorbs carbon dioxide from air. Keep well closed. Sol in water, lower alcohols, chloroform. Practically insol in ether, benzene, petr ether.
Diphosphate hexahydrate. $C_{10}H_{26}N_4O_8P_2 \cdot 6H_2O$, spermine phosphate. Crystal from water, mp 230-234° (dec). Sol in water 0.037% at 20°, 15% at 100°. Insol in alc, ether and other organic solvents. Sol in dilute acid and alkali. Known as Charcot-Neumann crystals, also found in sperm, blood, bone marrow in leukemia and secretions in asthma.
Tetrahydrochloride. $C_{10}H_{30}Cl_4N_4$, crystal from ethanol, mp 315-316°.
USE As a tool in biochemical research.

6897. Sperm Oil. From sperm whale, *Cetus*. Esters of fatty acids, small quantities of spermaceti.
Yellow, thin liquid, slight fishy odor if not of good quality. d 0.875-0.885. Refrac no. 1.29-1.47. Iodine no. 68-84. Insoluble in water, cold alcohol, in petr ether; sol in chloroform, ether.
USE As lubricant, in tanning, hardening steel, mineral soap.

6898. Sphenylglycine. *N-[4-(Aminoiminomethyl)-N-(2-methyl-2-butenyl)aspartic sphenylglycine*. $C_{20}H_{30}N_6$; mol wt 358.31. C 60.57%, H 11.35%, N 28.25%. Obtained from sperm oil. Isolated from the following: Central Asiatic *Leguminosae*: *Sarmiensa scheele* (Pall.) Turk. *Sphaerophysa salsula* (Pall.) DC.). Sarmiensa carduchorum Bunge, Sarmiensa flaccifolia Lehm.; Rubiaceae, Mendoza. *J Gen Chem USSR* 16, 561 (1946). Wijsbeem, *Ibid* 21, 2365 (1947); Studia, *Ibid* 22, 347 (1952). Structure: Hessing, Lebaid. *Ibid* 10, 534 (1970).



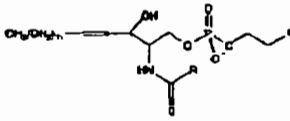
Strong, caustic base.
Dihydrochloride. $C_{20}H_{30}N_6O_2$, crystals, mp 149-150°.

6899. Sphingadiene. *[R-[R*,S*-(E)]]-2-Amino-1-octadecene-3,5-diol*; *(E)-2-amino-1-octadecene-3,5-diol*. 1,3-dihydroxy-2-amino-4-octadecene, 4-sphingenine. $C_{18}H_{35}NO_2$; mol wt 299.50. C 72.19%, H 12.43%, N 4.68%, O 10.68%. Important membrane component, long-chain amino dialcohol, mainly of esters, phosphatides, such as sphingomyelins, cerebrosides, and gangliosides. Material synthesized in D(+)-erythro-2,3-dihydroxy-2-amino-4-trans-octadecene. Does not exist in the free state in animals, plants or microorganisms. First obtained by hydrolysis of cerebrosides from brain: J. L. W. Thudichum, *Die Konstitution des Gehirns des Menschen und der Tiere* (Tübingen, 1901). Separation procedure: Carter et al., *J Biol Chem* 216, 249

Sphingosine C

Sphingolipid in C. $C_{21}H_{41}NO_4$, 3-keto-2-amino-1,4,14-octadecanetriol monoacetate. Cream colored solid

6900. Sphingomyelins. Sphingosine phosphatides occurring in the myelin sheaths of nerves. Found of beef heart sphingomyelin. Rapport, Loiron, *J Biol Chem* 180, 69 (1950). Isolation from human and rat brain: Hawthorne et al., *Nat Chim Acta* 46, 601 (1967); Thomson, Fujino, *J Biochem (Japan)* 59, 45 (1952); Rouser et al., *J Am Chem Soc* 73, 310 (1951); Maennes et al., *Ibid* 313. Structure of heavy bar (*Bos taurus*) sphingomyelin: Karlsson et al., *Biochim Biophys Acta* 270, 179 (1972). Synthesis of dihydrosphingomyelin: Shapiro et al., *J Am Chem Soc* 80, 2339 (1958). Synthesis of sphingomyelins: Shapiro et al., *Ibid* 81, 3743, 4350 (1959). Cerebrosides: Shapiro, *Flowers, Ibid* 84, 4047 (1962). Monograph: S. R. Kaery, *The Biology of Myelin* (Hoeber-Harper, New York, 1959).

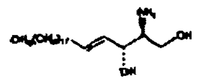


R = fatty acids such as stearic, palmitic, lignoceric, nervonic acids

Slightly hygroscopic powder. Occurs as a hydrate. Sol in hot absolute alcohol, acetic acid, chloroform, slightly sol in pyridine. Practically insol in ether, acetone, water.

Consult the Name Index before using this section

(1947), Willerding, ibid. 316, 329 (1951). Typian, Bioch Prep. 9, 121 (1962). Stereospecific synthesis of D-erythro-trans form and isomers: C. A. Grob, F. Gadient, Helv. Chim. Acta 40, 1145 (1957). Stereoselective synthesis: M. Newman, J. Am. Chem. Soc. 95, 4098 (1973); B. Bernet, A. Vasella, Tetrahedron Letters 24, 5491 (1983). Comprehensive review: C. A. Grob, Record Chem. Progr. (Kresge-Hooker Sci. Lib.) 18, 55-66 (1957); D. Shapiro, Chemistry of Sphingolipids (Hermann, Paris, 1969) 144 pp. Bibliography: Rodd's Chemistry of Carbon Compounds, Vol. I, part E, S. Coffey, Ed. (Elsevier, New York, 2nd ed., 1976) pp 394-391.



DL-Sphingosine, waxy crystals from ether + pentane, mp 67°.

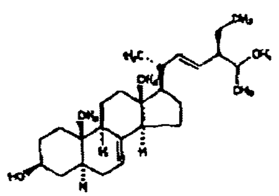
DL-Triacetylsphingosine, $C_{24}H_{43}NO_5$, crystals from pentane + ether, mp 91-92°.

Triacetyl derivative of natural sphingosine, $C_{24}H_{43}NO_5$, crystals, mp 101-102°. $[\alpha]_D$ −11.7° (chloroform).

8900. Spigelia. Pinkroot, Indian pink, Carolina pink, Maryland pink, wormgrass. Dried rhizome and roots of Spigelia marilandica L., Loganiaceae. Habit. North America (New Jersey to Florida and west to Wisconsin). Constit. Spigeline, resin, tannin, bitter principle, volatile oil.

THERAP CAT: Anthelmintic.

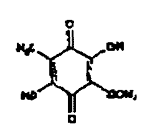
8901. α-Spinasterol. (3β,5α,22E)-Stigmasta-7,22-dien-3-ol; α-spinasterin; bessisterol; hitodesterol. $C_{29}H_{48}O$; mol wt 412.70. C 84.40%, H 11.72%, O 3.88%. Stereoisomeric with chondrillasterol: q.v. Extracted from spinach leaves: Hart, Heyl, J. Biol. Chem. 95, 311 (1932), from Citrullus colocynthis Schrad., Cucurbitaceae: Hamilton, Kermack, J. Chem. Soc. 1952, 5051. Structure: Fieser et al., J. Am. Chem. Soc. 71, 2226 (1949). Prepn: Barclay, Rosenstein, J. Org. Chem. 36, 2234 (1971); M. Anastasia et al., J. Chem. Soc. Perkin Trans. I 1983, 2365.



Crystals from alcohol + light pet. ether, mp 168-169°. $[\alpha]_D$ −3.6° (c = 2.8 in chloroform).

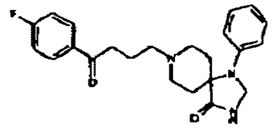
Benzoate, $C_{36}H_{52}O_2$, plates from ethyl acetate, mp 200°. $[\alpha]_D$ +1.6° (c = 1.7 in chloroform).

8902. Spinulosin. 3,6-Dihydroxy-2-methoxy-5-methyl-2,5-cyclohexadiene-1,4-dione; 2,5-dihydroxy-3-methoxy-6-methyl-p-benzoquinone; 3,6-dihydroxy-5-methoxy-p-toluquinone; 3,6-dihydroxy-4-methoxy-2,5-toluquinone; hydroxyfumigatin. $C_8H_8O_5$; mol wt 184.15. C 52.18%, H 4.38%, O 43.44%. Metabolic product of the fungi Penicillium spinulosum Thom and Aspergillus fumigatus Fres.: Birkinshaw, Raistrick, Phil. Trans. B220, 245 (1931); Anslow, Raistrick, Biochem. J. 32, 2288 (1938); Pettersson, Acta Chem. Scand. 17, 1771 (1963). Structure: Anslow, Erdtman, Svensk Kem. Tidskr. 48, 201 (1937). Synthesis: Anslow, Raistrick, Biochem. J. 32, 803 (1938). Biosynthesis: Pettersson, Acta Chem. Scand. 19, 1016 (1965).



Purple-black crystals, mp 203°. Slightly sol in cold water, more sol in hot water, sol in dil NaOH soln giving a purple soln with a bluish hue.

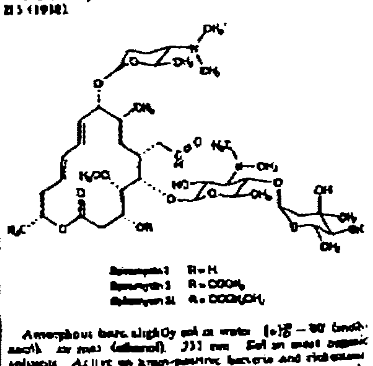
8903. Spiperone. 8-[4-(4-Fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one; 8-[3-(p-fluorobenzoyl)propyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one; 4'-fluoro-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]dec-8-yl)butyrophenone; Spiropitan. $C_{23}H_{26}FN_3O_2$; mol wt 395.48. C 69.85%, H 6.63%, F 4.80%, N 10.63%, O 8.09%. Prepn: Janssen, U.S. pats. 3,155,669; 3,155,670 and 3,161,644 (all 1964 to Janssen).



Crystals, mp 199-203°.

THERAP CAT: Antipsychotic.

8904. Spiramycin. RP-5337; Selectomycin; Rovamycin; Rovamycin. Antibiotic substance classified in the erythromycin-carbomycin group and produced by Streptomyces ambofaciens from soil of northern France: Corbaz et al., Compt. Rend. Soc. Biol. 226, 1491 (1952). Pinnert-Sindico et al., Antibiot. Ann. 1954-1955, 724. Ninet, Verrier, U.S. pat. 2,943,023 (1960 to Rhône-Poulenc); see also U.S. pat. 3,000,785 (1961 to Rhône-Poulenc). Antibacterial activity and toxicity: H. Sous et al., Antibiotiques Franch. 4, 366 (1955). Separation into 3 components named spiramycins I, II and III: Pinnert-Sindico, Charpentier, U.S. pats. 2,978,380 and 3,011,947 (1961 to Rhône-Poulenc). Structure: Kuehne, Benson, J. Am. Chem. Soc. 87, 4660 (1965). Revised structure: Omura et al., ibid. 91, 3401 (1969); Maezawa et al., J. Antibiot. 26, 55 (1973). Revised configuration at C-9: Frolich et al., J. Org. Chem. 39, 2474 (1974). Symposium on pharmacology, antibacterial spectrum, and clinical efficacy: J. Antimicrob. Chemother. 22, Suppl. B, 1-213 (1988).



Spiramycin I R = H
Spiramycin II R = $COCH_3$
Spiramycin III R = $COCH_2CH_3$

Amorphous base, slightly sol in water. $[\alpha]_D$ −80° (methanol). uv max (ethanol): 231 nm. Sol in most organic solvents. Active vs gram-positive bacteria and rickettsiae. Cross resistance between microorganisms resistant to erythromycin and carbomycin. LD_{50} in rats (mg/kg): 9400 orally, 1800 s.c., 170 i.v. (Sous).

Embonate: Spira 306.

Hemiadipate: spiramycin adipate, Stovamycin, Sequamycin.

Spiramycin I, $C_{43}H_{74}N_2O_{14}$, Foromacidin A. Crystals, mp 134-137°. $[\alpha]_D$ −96°.

Spiramycin I triacetate, crystals, mp 140-142°. $[\alpha]_D$ −87.5°.

Spiramycin II, $C_{45}H_{76}N_2O_{15}$, Foromacidin B. Crystals, mp 130-133°. $[\alpha]_D$ −86°.

Spiramycin II diacetate, crystals from cyclohexane, mp 151-160°. $[\alpha]_D$ −98.4°.

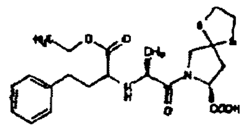
Spiramycin III, $C_{46}H_{78}N_2O_{15}$, Foromacidin C. Crystals, mp 128-131°. $[\alpha]_D$ −83°.

Spiramycin III diacetate, crystals from cyclohexane, mp 140-142°. $[\alpha]_D$ −90.4°.

THERAP CAT: Antibacterial.

THERAP CAT (VET): Antibacterial; growth promotant.

8905. Spirapril. (8S)-7-[(S)-N-[(S)-1-Carboxy-3-phenylpropyl]alanyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid 1-ethyl ester; (8S)-7-[(S)-N-[(S)-1-carboxy-3-phenylpropyl]alanyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid 1-ethyl ester. $C_{22}H_{30}N_2O_5S_2$; mol wt 466.62. C 56.63%, H 6.48%, N 6.00%, O 17.14%, S 13.74%. Angiotensin-converting enzyme (ACE) inhibitor. Prepn: E. H. Gold et al., U.S. pat. 4,470,972 (1984 to Schering); E. M. Smith et al., J. Med. Chem. 32, 1600 (1989). Pharmacology: E. J. Sybertz et al., J. Pharmacol. Exp. Ther. 246, 214 (1987); T. Baum et al., ibid. 230 Radioimmunoassay in plasma: M. Hoover-Nix et al., Ther. Drug Monit. 14, 224 (1992). Pharmacokinetics and evaluation in CHF: S. A. J. van den Broek et al., J. Cardiovasc. Pharmacol. 16, 614 (1991). Clinical evaluation in essential hypertension: O. F. Ramos et al., J. Clin. Pharmacol. 31, 546 (1991). Review: V. Jain, H.-J. Kramer, Cor Vasa 34, 12-81 (1992).



Hemihydrate, white foam. $[\alpha]_D$ −29.5° (c = 0.2 in ethanol).

Hydrochloride, $C_{22}H_{30}N_2O_5S_2.HCl$, Sch-33844, TI-211-950, Renormax, Renpress, Sandopril. White solid, mp 192-194° (dec). $[\alpha]_D$ −11.7° (c = 0.4 in ethanol).

Diacid, $C_{20}H_{26}N_2O_5S_2$, spiraprilat, spiraprilic acid, Sch-33861. Monohydrate, mp 163-165°. $[\alpha]_D$ +4.1° (c = 0.4 in ethanol).

THERAP CAT: Antihypertensive.

8906. Spirit of Ammonia, Aromatic. Spirit of hartshorn, aromatic. A soln contg 34 g ammonium carbonate, 90 ml 10% ammonia water, 10 ml lemon oil, 1 ml oil of lavender, 1 ml oil of myristica, 700 ml alcohol and a sufficient quantity of water to make 1 liter. Alcoholic alc by vol. ~65%.

Almost colorless to slightly yellow liquid with an aromatic, pungent odor and the taste of ammonia. d 0.90. Forms an opalescent mixture with water, miscible with alcohol.

THERAP CAT: Reflex respiratory stimulant.

THERAP CAT (VET): By inhalation: respiratory and cerebral stimulant. Internally: expectorant, stomachic, antacid, carminative. Externally: counterirritant, and to alleviate the irritation of insect stings and bites.

8907. Spirit of Camphor. A soln of camphor in alcohol contg 10 g camphor per 100 ml soln.

Colorless liquid, camphor odor.

THERAP CAT: Counterirritant, analgesic (topical), antipruritic.

THERAP CAT (VET): Has been used as rubefacient, stimulant, expectorant, carminative.

8908. Spirit of Chloroform. An alcoholic soln of chloroform contg 6% by vol of chloroform, corresponding to 10.5% by wt, and ~89% abs alcohol by vol.

Colorless, clear liquid, chloroform odor. d ~0.85.

THERAP CAT: Carminative.

8909. Spirit of Ether. Hoffmann's Drops. A mixture of alcohol and ether. Made by mixing 325 ml ether U.S.P. with sufficient U.S.P. alcohol (~690 ml) to make 1000 ml. Absolute alc by vol. ~66%.

Colorless, volatile liquid. d 0.765.

THERAP CAT: Carminative.

8910. Spirit of Ether Compound. Hoffmann's anodyne. A mixture of 325 ml ether U.S.P., 25 ml ethereal oil and sufficient alcohol (~665 ml) to make 1000 ml. Absolute alc by vol. ~66%.

Colorless, clear liquid. d ~0.785.

THERAP CAT: Carminative.

THERAP CAT (VET): Has been used as a stomachic, carminative, in colic.

8911. Spirit of Ethyl Nitrite. Spirit of nitrous ether, sweet spirit of niter. An alcoholic soln of ethyl nitrite contg 3.5-4.5% ethyl nitrite. Incompat: Antipyrine, iron salts, acetanilide, acetophenetidin, iodides, tincture guaiac, morphine salts, carbonates, acacia.

Pale yellow or faintly greenish-yellow, clear, mobile, volatile, flammable liquid, ethereal, pungent odor, sharp burning taste. The ethyl nitrite evaporates rapidly; dec on exposure to air, dec in light. d not above 0.823 at 25°. Miscible with water and alcohol. Keep tightly closed, protected from light, in a cool place, remote from fire.

THERAP CAT: Diaphoretic, diuretic.

8912. Spirit of Formic Acid. Spirit of ants. Composed of 40 ml of 75% formic acid and 335 ml water in sufficient alcohol to make one liter; corresponds to ~1% formic acid and ~70% abs alcohol by vol.

Colorless liquid. Miscible with water, alcohol.

8913. Spirit of Glyceryl Trinitrate. Spirit of nitroglycerin, spirit of trinitroglycerin, spirit of glonoin, soln. of glyceryl trinitrate. An alcoholic soln contg 1.0-1.1% glyceryl trinitrate. Incompat: Alkalies, carbonates, HCl, HI.

Colorless, clear liquid. d 0.814-0.820. Miscible with alcohol, chloroform, ether; 1 ml dissolves in 6 ml almond oil; very slightly sol in water, miscible with chloroform, ether.

Caution: Likely to produce violent headache when tasted or applied to the skin.

Note: When spilled, immediately pour NaOH soln over it or dry residue may explode by friction or shock.

THERAP CAT: Vasodilator.

8914. Spirit of Peppermint. An alcoholic soln contg per liter 100 ml oil of peppermint and the alcohol-soluble principles from 10 g of powdered peppermint previously macerated with water.

THERAP CAT: Carminative.

THERAP CAT (VET): Has been used as a carminative.

8915. Spirit of Spearmint. An alcoholic soln contg per liter 100 ml oil of spearmint and the alcohol-soluble principles from 10 g coarsely powdered spearmint leaves previously macerated with water.

THERAP CAT: Carminative.

THERAP CAT (VET): Has been used as a carminative.

8916. Spirogermanium. 8,8-Diethyl-N,N-dimethyl-2-aza-8-germaspiro[4.5]decane-2-propanamine; 2-[3-(dimethylamino)propyl]-8,8-diethyl-2-aza-8-germaspiro[4.5]decane. $C_{17}H_{36}GeN_2$; mol wt 341.10. C 59.86%, H 10.64%, Ge 21.29%, N 8.21%. Cytotoxic germanium deriv. Prepn: L. M. Rice, Ger. pat. 2,342,348; eidem, U.S. pat. 3,825,546 (1973, 1974 both to Unimed); Rice et al., J. Heterocycl. Chem. 11, 1041 (1974). Preclinical toxicological evaluation: M. C. Henry, C. D. Port, U.S. NTIS Rep. PB-264173 (1977) 213 pp; C.A. 87, 177630 (1977). Early clinical studies: P. S. Schein et al., Cancer Treat. Rep. 64, 1051 (1980); C. Trope et al., ibid. 65, 119 (1981). Toxicity study: M. C. Henry et al., ibid. 64, 1207 (1980).

Consult the Name Index before using this section

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[illegible]

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

7590 09/23/2003

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

EXAMINER
WITZ, JEAN C

ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 09/23/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

Notice of Abandonment	Application No.	Applicant(s)	
	09/830,146	BEAUDOIN ET AL.	
	Examiner	Art Unit	
	Jean C. Witz	1651	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on *25 February 2003*.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 (a) to the final rejection.
   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114).
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☒ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $_____ is insufficient. A balance of $_____ is due.
   The issue fee required by 37 CFR 1.18 is $_____. The publication fee, if required by 37 CFR 1.18(d), is $_____.
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☐ The reason(s) below:

Jean C. Witz
Primary Examiner
Art Unit: 1651

Petitions to revive under 37 CFR 1.137(a) or (b), or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/830,146	07/25/2001	Adrien Beaudoin	789-47	9803

7590 02/25/2003

Akerman Senterfitt & Eidson
Post Office Box 3188
West Palm Beach, FL 33402-3188

EXAMINER
WITZ, JEAN C

ART UNIT	PAPER NUMBER
1651	

DATE MAILED: 02/25/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

Office Action Summary	Application No. 09/830,146	Applicant(s) BEAUDOIN ET AL.	
	Examiner Jean C. Witz	Art Unit 1651	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *20 November 2002* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,4-12,14-17,19-27,30-33 and 37-51* is/are pending in the application.

4a) Of the above claim(s) *26,33,45 and 46* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,4-12,14-17,19-25,27,30-32,37-44 and 47-51* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) *6&7* .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Claim Objections

The numbering of claims is not in accordance with 37 CFR 1.126 which requires the original numbering of the claims to be preserved throughout the prosecution. The original claims did not include claim 2 or claim 3. When claims are canceled, the remaining claims must not be renumbered. When new claims are presented, they must be numbered consecutively beginning with the number next following the highest numbered claims previously presented (whether entered or not).

The misnumbered claims 4-51 have not been renumbered. This is due to the fact that the misnumbering of claims was not discovered until after the preliminary amendment filed April 20, 2001 in which original claims 13, 18, 28-29, and 32-33 were been cancelled. As a result, renumbering of the claims would render the file too confusing as to whether cancelled claims should be renumbered along with the need to further renumber the dependency in the dependent claims. Therefore, all claim numbers referred to in this office action represent original claim numbers.

However, as a result, these claims are objected for the reasons set forth above. It is strongly suggested that in response to this office action, Applicants cancel all pending claims and submit new claims with proper numbering and in proper order to represent the invention starting with claim 52.

Election/Restrictions

Applicant's election of Group I, claims 1, 4-12, 14-17, 19-25, 27, 30-32, 36-44 and 47-51 in Paper No. 9 is acknowledged. Because applicant did not distinctly and

specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claims 26, 33 and 45-46 are withdrawn from further consideration pursuant to 37 CFR 1.142(b).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1, 4-12, 14-17, 19-25, 27, 30-32, 36-44, 47-51 are rejected under 35 U.S.C. 103(a) as being unpatentable over the disclosures of CA 2,155,571, WO 8401715, JP 360035057A and Collin (U.S. 6,055,936) taken as a whole.

The cited claims are drawn to the extraction of lipids from marine and aquatic animals material comprising the extraction of the material in a ketone such as acetone, the separation of liquid and solid phases, the evaporation of the solvent from the liquid phase, the re-extraction of the solid phase in a solvent such as an alcohol, the separation of the liquid and solid phase, the evaporation of the solvent from the second liquid phase and the recovery of the solid phase. The solid phase contains active enzymes.

Each step in this extraction process is well known in the art. Both JP 360035057A and Collin disclose that acetone is a conventional extractant for marine animals for the recovery of lipid pigments containing astaxanthin and canthaxanthin. CA 2,155,571 discloses that lower alcohols such as ethanol are known to extract long chain polyunsaturated fatty acids from marine organisms. Finally, WO 8401715 discloses that after lipid extraction of krill, the remaining solid phase contains active enzymes.

Therefore, it would have been obvious to one of ordinary skill in the art at the time the invention was made to extract known components from marine and aquatic animals with the reasonable expectation of obtaining these components. The motivation to combine steps flows from the desire to maximize the number of different useable components from a single source within a single procedure. Dependent claims reciting specific extraction, separation, and drying parameters represent procedures well known in the art. For example, finely dividing any material to be extracted is well known in order to maximize the surface area of the material to the extracting liquid.

Further, maintenance of extraction temperatures at or below 5 degrees C is well known and performed in order to reduce oxidation of the fatty acids contained in the material. Drying and separation techniques claimed are well known and conventional in the art.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jean C. Witz whose telephone number is (703) 308-3073. The examiner can normally be reached on 6:30 a.m. to 4:00 p.m. M-Th and alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Wityshyn can be reached on (703) 308-4743. The fax phone numbers for the organization where this application or proceeding is assigned are (703) 872-9306 for regular communications and (703) 872-9307 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Jean C. Witz
Primary Examiner
Art Unit 1651

February 24, 2003

Notice of References Cited	Application/Control No. 09/830,146	Applicant(s)/Patent Under Reexamination BEAUDOIN ET AL.	
	Examiner Jean C. Witz	Art Unit 1651	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-6,055,936	05-2000	Collin	11/215
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	360035057	02-1985	JP	Yasuda	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Mark D. Passler, Reg. No. 40,764

Form PTO-1449 (Rev. 2-88)

U.S. DEPARTMENT OF COMMERCE
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ATTY. DOCKET NO.	APPLICATION NO.
789-47	09/830,146

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use several sheets if necessary)

APPLICANT: BEAUDOIN, *et al.*

FILING DATE: April 20, 2001

GROUP:

U.S. PATENT DOCUMENTS

EXAMINER'S INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
		4,331,695	5/25/82	Zosel			
		5,006,281	4/9/91	Rubin, et al.			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	✓	2,115,571	1993	Canada				
	✓	1098900	4/7/81	Canada				
	✓	JP 08198754	6/8/96	Japan				X
	✓	WO 8401715	5/10/84	WO				
	✓	JP 04057853	2/25/92	JP				X
	✓	EP 0732378	9/18/96	EP				
	✓	NO 147365	5/28/82	NO				X

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

	✓	Patent Abstracts of Japan, 2 July 1976, JP 51 076467
	✓	Patent Abstracts of Japan vo. 009, no. 059, 15 March 1985, JP 59196032
	✓	Thomas Carell, Edward A. Wintner, A. Bashir-Hashemi, Julius Rebek, Jr. "A Novel Procedure for the Synthesis of Libraries Containing Small Organic Molecules". Angew. Chem. Int. Ed. Engl. 1994, 33, No. 20; page 2059.
	✓	Thomas Carell, Edward A. Wintner, Julius Rebek, Jr. "A Solution-Phase Screening Procedure for the Isolation of Active Compounds from a Library of Molecules". Angew. Chem. Int. Ed. Engl. 1994, 33, No. 20; page 2061.
	✓	Marie-Thérése Château, Céline Ginestier-Verne, Jean Chiesa, René Caravano, Jean Paul Bureau. "Dimethyl sulfoxide-induced apoptosis in human leukemic U937 cells". Analytical Cellular Pathology 10 (1996) 75-84.
		SEE ATTACHED SUPPLEMENTAL SHEET

EXAMINER: WTB

DATE CONSIDERED: 2/23/03

* EXAMINER: Initial if a citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

SUPPLEMENTAL SHEET TO
INFORMATION DISCLOSURE STATEMENT
BY APPLICANT

			OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)* Continued
		✓	Takashi Kojima, Masao Yamamoto, Chihiro Mochizuki, Toshihio Mitake, Norimasa Sawada and Yohichi Mochizuki. "Different Changes in Express and Function of Connexin 26 and Connexin 32 During DNA Synthesis and Redifferentitation in Primary Rat Hepatocytes Using a DMSO Culture System" Hepatology, September 1997; Vol. 26, No. 3, 1997
		✓	Kit S. Lam. "Application of conbinatorial library methods in cancer research and drug discovery". Anti-Cancer Drug Design (1997), 12, 145-167.
		✓	Jose Prados, Consolacion Melguizo, Juan Emilio Fernandez, Amelia Eva Aranega, Luis Alvarea and Antonia Arangea. "Actin, Tropomyosin and α-Actinin as Markers of Differentiation in Human Rhabdomyosarcoma Cell Lines Induced with Dimethyl Sulfoxide". Cellular Molecular Biology. 39(5) 525-536, 1993.
		✓	Howard M. Prentice, Stephen E. Moore, John G. Dickson, Patrick Doherty and Frank S. Walsh. "Nerve growth factor-induced changes in neural cell adhesion molecule (N-CAM) in PC12 cells". The EMBO Journal, Vol. 6, No. 7, pp. 1859-1863, 1987.
		✓	Stefan Sjolander and Csaba Urbaniczky, "Integrated Fluid Handling System for Biomolecular Interaction Analysis". Analytical Chemistry, Vol. 63, No. 29, October 15, 1991.
		✓	Alexander Szabo, Lesley Stolz and Russ Granzow.. "Surface plasmon resonance and its use in biomolecular interaction analysis (BIA)".
		✓	O. Trubiani, C. Peiri, M. Rapino, R. DiPrimo. "The *c-myc* gene regulats the polyamine pathway in DMSO-induced apoptosis". Cell Proliferation, 1999, 32, 119-129.
			R.A. Houghten, J.R. Appel, S.E. Blondelle, J.H. Curevo, C.T. Dooler and C. Pinilla. "The Use of Synthetic Peptide Combinatorial Libraries for the Identification of Bioactive Peptides" BioTechniques Vol. 13, No. 3 (1992)

WTT 2/23/03

Docket No. 789-47

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PATENT AND TRADEMARK OFFICE

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use several sheets if necessary)

ATTY. DOCKET NO.	APPLICATION NO.
789-47	09/830,146
APPLICANT: BEAUDOIN, *et al.*	
FILING DATE: April 20, 2001	GROUP:

U.S. PATENT DOCUMENTS

EXAMINER'S INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	✓	JP 08198754	8/6/96	Japan			x (ABSTRACT)	
	✓	JP 53112195	9/30/78	Japan			x (ABSTRACT)	
	✓	JP 60 035057	2/22/85	Japan			x (ABSTRACT)	
	✓	JP 04 057853	2/25/92	Japan			x (ABSTRACT)	

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

		✓	Chemical Abstracts no. 177859, vol. 98, no. 21 "Isolation of astaxanthin from crayfish or shrimp waste for use as a coloring agent in fish feed." 05/23/83

EXAMINER: WTT DATE CONSIDERED: 2/23/03

* EXAMINER: Initial if a citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

1662 10

PATENT

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GROUP 1600

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	BEAUDOIN et al.	Examiner:	Witz, Jean C.
Serial No.:	09/830,146	Group No.:	1651
Filed:	July 25, 2001		

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

OFFICIAL

TRANSMITTAL LETTER

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being transmitted to the Commissioner for Patents, Washington, DC 20231, via facsimile (fax no. 703-872 9306) on November 20, 2002.

Reg No. 40,764
Mark D. Passler

VIA FACSIMILE (703 872 9306)
Tech Center 1600

Total pages (including this one): 4

Commissioner for Patents
Washington, D.C. 20231

Sir:

Please find enclosed:

Response to Restriction Requirement
Request for Retroactive Extension of Time

Please charge the $55.00 extension fee to Deposit Account No. 500951. Please charge any additional fees, or credit any overpayments, to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 11/20/02

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
222 Lakeview Avenue, Suite 400
P. O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47
(WP11221;1)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	BEAUDOIN et al.	Examiner:	Witz, Jean C.
Serial No.:	09/830,146	Group No.:	1651
Filed:	July 25, 2001		

Title: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

RESPONSE TO RESTRICTION REQUIREMENT

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being transmitted to the Commissioner for Patents, Washington, DC 20231, via facsimile (fax no. 703-872 9306) on November 20, 2002.

Reg No. 40,764
Mark D. Passler

VIA FACSIMILE (703 872 9306)
Tech Center 1600

Commissioner for Patents
Washington, D.C. 20231

Sir:

This response is in reply to a restriction requirement set forth in the Office Action dated October 2, 2002, in the above-identified application, with a one month shortened statutory period, making a response due on or before November 2, 2002. This response is filed with a one month extension of time.

REMARKS

In the above-identified Office Action, the Examiner has issued a restriction requirement and requires election of one of the following species under 35 U.S.C. § 121:

Group I: Claims 1-12, 14-17, 19-25, 27, 30-32, 36-44, and 47-51, drawn to a method lipid extraction;

Group II: Claims 26 and 45-46, drawn to a carotenoid composition; or

Group III: Claim 33, drawn to a lipid fraction.

Adjustment date: 04/17/2003 EEKUBAY1
12/06/2002 JWASHING 00000001 500951 09830146
01 FC:1251 110.00 CR

{WP112217;1}

12/06/2002 JWASHING 00000001 500951 09830146
01 FC:1251 110.00 CH

04/17/2003 EEKUBAY1 00000005 500951 09830146
01 FC:2251 55.00 CH

Applicant hereby elects the species of Group I (claims 1-12, 14-17, 19-25, 27, 30-32, 36-44, and 47-51) for further prosecution. Withdrawal f the outstanding restriction requirement under 35 U.S.C. § 121 and examination on the merits is respectfully requested.

Respectfully submitted,

Date: 11/20/02

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
222 Lakeview Avenue, Suite 400
P. O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket no. 789-47

{WP112217;1}

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	BEAUDOIN et al.	Examiner:	Witz, Jean C.
Serial No.:	09/830,146	Group No.:	1651
Filed:	July 25, 2001		
Title:	METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES		

REQUEST FOR RETROACTIVE EXTENSION OF TIME

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being transmitted to the Commissioner for Patents, Washington, DC 20231, via facsimile (fax no. 703-872 9306) on November 20, 2002.

Reg No. 40,764
Mark D. Passler

VIA FACSIMILE (703 872 9306)
Tech Center 1600

Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a request for a one month extension of time pursuant to 37 CFR §1.136. Please charge the fee of $55.00 and any deficiencies, and credit any overpayments, to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 11/20/02

J. Rodman Steele, Jr.
Registration No. 25,931
Mark D. Passler
Registration No. 40,764
Akerman Senterfitt
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

{WP112217;1}

(19)日本国特許庁（ＪＰ）　(12) 公開特許公報（Ａ）　(11)特許出願公開番号

特開平8－198754

(43)公開日　平成８年(1996)８月６日

(51)Int.Cl.[6]	識別記号	庁内整理番号	ＦＩ	技術表示箇所
Ａ６１Ｋ　31/66	ＡＡＭ			

審査請求　未請求　請求項の数５　ＦＤ　（全５頁）

(21)出願番号　特願平7－25822

(22)出願日　平成７年(1995)１月23日

(71)出願人　000006884
株式会社ヤクルト本社
東京都港区東新橋１丁目１番19号
(72)発明者　酒井　正士
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内
(72)発明者　工藤　聰
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内
(72)発明者　大和矢　秀行
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内
(74)代理人　弁理士　佐藤　正年　（外１名）

(54)【発明の名称】　脳機能改善剤

(57)【要約】
【目的】　本発明は、コスト面や供給面で問題がなく、脳機能改善を行なうことのできる脳機能改善剤を得る。
【構成】　ウシ脳以外のホスファチジル－Ｌ－セリン又はその塩を有効成分とするものである。

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【特許請求の範囲】

【請求項1】　ウシ以外の動物の脳由来のホスファチジルーLーセリン又はその塩を有効成分とする脳機能改善剤。

【請求項2】　前記ウシ以外の動物が、豚、羊、鶏である請求項1に記載の脳機能改善剤。

【請求項3】　綿実由来のホスファチジルーLーセリン又はその塩を有効成分とすることを特徴とする脳機能改善剤。

【請求項4】　酵母由来のホスファチジルーLーセリン又はその塩を有効成分とする脳機能改善剤。

【請求項5】　鶏肉，鶏内臓，魚体，魚肉の何れか由来のホスファチジルーLーセリン又はその塩を有効成分とする脳機能改善剤。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は例えば脳機能を改善させる組成物に関し、アルツハイマー病やパーキンソン病のような痴呆症の予防や治療に有効な組成物である脳機能改善剤に関するものである。

【0002】

【従来の技術】ブルニ(A.Bruni)らはウシの脳から抽出したホスファチジルセリンをマウスの尾静脈に注射すると、脳内グルコース濃度が対照群の約4倍に上昇することを報告している(Nature,vol.260,p.331,1976)。また、このウシの脳から抽出したホスファチジルセリンを12週間経口投与することによって、記憶力の低下を示した老齢ラットの行動を改善することが報告されている(A.Zanotti et al.,Psychopharmacology Berl.,vol.99,p316,1989)。

【0003】更に、ヒトでの臨床試験でも、ウシ脳ホスファチジルセリンがアルツハイマー病や老年期の記憶障害に有効なことが二重盲検－プラセボ試験で報告されている(P.J.Delwaide et al.,Acta Neurol.Scand.,vol.73,p.136,1986;R.R.Engel et al.,Eur. Neuropsychopharmacol.,vol.2,p.123,1993)。

【0004】このように脳内グルコース濃度の増加を示すウシ脳ホスファチジルセリンはラットやヒトの脳機能改善効果を示す。従って、脳内グルコース濃度の上昇は脳機能改善作用物質を選ぶ際の指標と考えられるが、一方では先程上げたブルニらの論文によれば大豆から抽出されたホスファチジルセリンには、このような作用はないと報告されており、脳機能の改善作用の発現にはホスファチジルセリン中の脂肪酸組成が重要と考えられている。

【0005】即ち、ウシ脳から抽出されたホスファチジルセリンは、その構成脂肪酸鎖について、1位にステアリン酸鎖，2位にオレイン酸鎖が多いと言う極めて特異的な脂肪酸鎖の構成を有しており、この特異な構成脂肪酸鎖を有することが脳機能の改善作用の発現に必要であると考えられていた。

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【0006】一方、最近、合成法で得られた特定の脂肪酸組成を有するホスファチジルセリンがプロテインキナーゼCアイソザイムの活性化作用に基づき、老人性痴呆症に対しての用途が期待できるとした特許出願が行われているが、インビボ(in vivo)で有効性を確認したものではない（特開平6－279311号公報）。

【0007】一方、リゾ型のホスファチジルセリンについては、ウシ脳ホスファチジルーLーセリンから誘導された、リゾホスファチジルーLーセリンが、脳内或いは血中グルコース上昇作用を示すことが報告されている(H.W.Chang et al.,Br.J.Pharmacol.,vol.93,p.611,1988)。

【0008】このように、従来は、ウシ脳から抽出されたホスファチジルセリンがその特徴的な構成脂肪酸の組成に基づき、脳内グルコース上昇作用を示すと考えられていた。

【0009】

【発明が解決しようとする課題】前述のように、従来の文献等では、ウシ脳から抽出されたホスファチジルセリン或いはそのリゾ型についてしか脳内グルコース上昇作用は知られていなかった。しかしながら、ウシ脳から抽出されたホスファチジルセリンはウシ1頭分の脳から約1gしか得られないので、コスト面でも、量的供給面でも大きな制限があることは明白である。

【0010】本発明者らは、鋭意研究の結果，ウシ脳由来のホスファチジルセリン以外にも、ウシ以外の動物の脳由来、獣肉や魚肉由来、内臓由来、植物由来、或いは微生物由来のホスファチジルーLーセリンにも脳機能改善効果（記憶障害回復効果）があることを見出し、ウシ脳以外に由来するホスファチジルセリンも抗痴呆物質として利用できることを明らかにして、本発明を完成した。

【0011】即ち、本発明は、コスト面や供給面で問題がなく、脳機能改善を行なうことのできる脳機能改善剤を得ることを目的とする。

【0012】

【課題を解決するための手段】本請求項1に記載された発明に係る脳機能改善剤では、ウシ以外の動物の脳由来のホスファチジルーLーセリン又はその塩を有効成分とするものである。

【0013】本請求項2に記載された発明に係る脳機能改善剤では、請求項1に記載されたウシ以外の動物が、豚、羊、鶏であるものである。

【0014】本請求項3に記載された発明に係る脳機能改善剤では、綿実由来のホスファチジルーLーセリン又はその塩を有効成分とするものである。

【0015】本請求項4に記載された発明に係る脳機能改善剤では、酵母由来のホスファチジルーLーセリン又はその塩を有効成分とするものである。

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【００１６】本請求項５に記載された発明に係る脳機能改善剤では、鶏肉，鶏内臓，魚体，魚肉の何れか由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするものである。

【００１７】

【作用】本発明の脳機能改善剤では、ウシ以外の動物の脳由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするものであるため、脳内グルコース濃度を増加させることができる。従って、投与された被験体の脳機能を改善する効果を有する。また、具体的なウシ以外の動物としては、豚，羊，鶏等が上げられる。

【００１８】また別の本発明の脳機能改善剤では、綿実由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするもの、酵母由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするもの、更に、鶏肉，鶏内臓，魚体，魚肉の何れか由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするものであるため、同じく、脳内グルコース濃度を増加させることができる。従って、投与された被験体の脳機能を改善する効果を有する。

【００１９】具体的には、鶏内臓としては、鶏肝臓が上げられる。また、魚体，魚肉としては、イワシ，マグロ，サバ等の魚体，魚肉，血合肉，また、魚油の絞り粕，魚の内臓，魚の内臓から肝油を絞った残渣等が上げられる。

【００２０】以上のように、本発明に係る脳機能改善剤では、ウシ以外の動物の脳由来、獣肉や魚体，魚肉由来、内臓由来、植物由来、或いは微生物由来のホスファチジル－Ｌ－セリン又はその塩を有効成分とするものである。このウシ脳以外のホスファチジル－Ｌ－セリンは、後述する脂肪酸鎖の組成を有しており、脂肪酸鎖の組成と脳機能改善効果（脳内グルコース濃度の上昇効果）との関係は必ずしも明確でないが、少なくとも、後述する実施例で効果を確認した範囲では、有効であることが認められた。

【００２１】また、本発明のウシ脳以外のホスファチジル－Ｌ－セリンの塩は、薬学上許容し得る塩の形で用いればよい。具体的には、ナトリウム塩，カリウム塩，マグネシウム塩，アンモニウム塩，リン酸塩，塩酸塩，硫酸塩等があるが、ナトリウム塩，カリウム塩が好ましい。

【００２２】更に、本発明のウシ脳以外のホスファチジル－Ｌ－セリン又はその塩の投与は、静脈内投与でも経口投与でも有効である。また、他の脂質、糖、タンパク質等の賦形剤を混ぜて、扱い易さや保存性を向上させたカプセル状や顆粒剤に加工しても良い。更に、安全性の点でも問題がないので、日常摂取する飲食品中に配合し、脳機能障害の軽減や予防に使用することもできる。

【００２３】本発明に係るウシ脳以外のホスファチジル－Ｌ－セリン又はその塩は、原料となる各種動物組織

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や、綿実レシチン，酵母等から常法の有機溶媒を用いた精製操作によって、ホスファチジル－Ｌ－セリンを製造することができる。原料として用いる各種動物組織や、綿実レシチン，酵母等については、何れもウシ脳に比べてはるかに大量にまた安価に提供可能であり、また供給面での量的問題も少ない。

【００２４】また、用いる各種動物組織や、綿実レシチン，酵母由来のホスファチジル－Ｌ－セリンは適当な精製処理工程に付し、不純物を除いて用いることが望ましいが、投与上の問題や効果を阻害するような問題がない限り、原料由来や生成工程での不純物を含んだまま用いても良い。

【００２５】

【実施例】

実施例１．動物組織からのホスファチジル－Ｌ－セリンの精製

動物組織からのホスファチジル－Ｌ－セリンの精製は、「新生化学実験講座４脂質II　リン脂質（東京化学同人，1991）8.3 ホスファチジルセリン（新井洋由）」を参考に行った。動物組織（ブタ脳，ヒツジ脳，鶏肉，鶏肝臓、イワシ魚体，サバ血合肉）を細かく切断し、200ｇ当り、60mlのアセトンを加え、ワーリングブレンダーでホモジナイズした。これにアセトン 200mlを加え、上清を吸引濾過して除いた残渣を800mlのアセトン、400mlのエタノールで洗浄後、800mlの石油エーテルで一晩撹拌しながら脂質を抽出した。抽出物を減圧乾固して、10mlのエチルエーテルに溶解し、ここに 100mlのエチルアルコールを徐々に加え、吸引濾過により沈殿を回収した。回収した沈殿物を減圧乾固し、そのうちの５ｇに対してクロロホルムを加えて溶解したものをシリカゲル（Silica gel 60, MERCK社製）を充填したカラム（φ32mm× 300mm）にホスファチジル－Ｌ－セリンを含む画分を分取した。シリカゲル薄層クロマトグラフィーにより分析した結果、各精製品のホスファチジル－Ｌ－セリン含量は90％以上であった。

【００２６】実施例２．綿実レシチンからのホスファチジル－Ｌ－セリンの精製

綿実からのホスファチジル－Ｌ－セリンの精製も、「新生化学実験講座４　脂質II　リン脂質（東京化学同人，1991）8.3 ホスファチジルセリン（新井洋由）」を参考に行った。綿実レシチン５ｇに対してクロロホルムを加えて溶解したものをシリカゲル（Silica gel 60, MERCK社製）を充填したカラム（φ32mm× 300mm）を用いて精製してスファチジル－Ｌ－セリンを含む画分を分取した。シリカゲル薄層クロマトグラフィーにより分析した結果、各精製品のスファチジル－Ｌ－セリン含量は90％以上であった。

【００２７】実施例３．各種ホスファチジル－Ｌ－セリンの脂肪酸鎖の組成

実施例１，２で得られた各種ホスファチジル－Ｌ－セリ

5　　6

ンの脂肪酸鎖の組成の分析を行った。具体的には、「生物化学実験法９脂質分析法入門（藤野康彦著、学会出版センター）」に従って、メチルエステル化した試料をキャピラリーＧＬＣにより分析した。結果を以下の表１に示す。尚、表中、「ＰＳ」はホスファチジルーＬーセリ＊ンを、「16:0」はパルミチン酸，「18:0」はステアリン酸，「18:1」はオレイン酸，「18:2」はリノール酸，「20:4」はアラキドン酸を示す。

【００２８】

【表１】

	16:0	18:0	18:1	18:2	20:4	その他
ウシ脳ＰＳ	1.5	49.9	30.4	-	1.2	17.0
ブタ脳ＰＳ	1.2	48.1	32.5	-	1.5	16.7
ヒツジ脳ＰＳ	1.8	47.1	30.5	-	1.7	18.9
鶏肉ＰＳ	13.8	3.9	10.2	63.2	5.9	3.0
鶏肝臓ＰＳ	35.9	8.8	7.5	40.0	3.1	4.7
イワシ魚体ＰＳ	20.3	2.1	15.3	2.5	2.2	57.6
サバ血合肉ＰＳ	18.6	5.6	8.2	1.1	3.0	63.5
綿実ＰＳ	32.3	0.5	14.2	50.6	-	2.2
酵母ＰＳ（S.cerebisiae）	42.5	3.4	35.7	-	-	18.4

【００２９】表１に示すように、ウシ脳から抽出されたホスファチジルーＬーセリンの脂肪酸はステアリン酸とオレイン酸とが大半を占める。これに対して、ブタ脳及びヒツジ脳由来のホスファチジルーＬーセリンの脂肪酸も同様な構成であり、このステアリン酸とオレイン酸とが大半を占める構成は、動物の脳自体に特有の脂肪酸の構成であることが推測された。

【００３０】ところが、鶏肉，鶏肝臓，サバ血合肉，綿実酵母由来のホスファチジルーＬーセリンの脂肪酸は、ウシ脳及び他の動物脳由来及び酵母由来のホスファチジルーＬーセリンの脂肪酸の構成とはかなり異なることが判った。

【００３１】鶏肉及び鶏肝臓由来のホスファチジルーＬーセリンの脂肪酸では、リノール酸が多く、サバ血合肉由来のホスファチジルーＬーセリンの脂肪酸では、パルミチン酸，ステアリン酸，オレイン酸，リノール酸，アラキドン酸以外の脂肪酸が大半を占める。また、綿実由来のホスファチジルーＬーセリンの脂肪酸は鶏肝臓由来のものと似て、リノール酸とパルミチン酸とが大半を占める。更に、酵母由来のホスファチジルーＬーセリンの脂肪酸は、パルミチン酸とオレイン酸とが大半を占める。

【００３２】実施例４．スコポラミン誘発記憶障害の改善

各群10頭の雄性ラット（ＳＤ系，体重約 300ｇ）にスコポラミン溶液（ 3.0mg/ml 緩衝液）及び各種ＰＳ溶液（60mg/ml 緩衝液）をそれぞれ 1.0ml/kg ずつ腹腔内に投与し、投与20分後にラット用ステップスルー・ケージ（室町機械（株））の明室におき、約10秒後に明室と暗室とを仕切るドアを開けてラットが暗室に入った直後に２秒間の電気刺激（４mA,100Ｖ，直流）を与えた。そして、投与後24時間後に再びラットを明室におき、四肢が暗室に入るまでの時間（反応潜時）を最大５分間まで計測した。この反応潜時が長いほど、電気刺激を受けた経験を良く記憶していると判断される。

【００３３】

【表２】

7　　8

	暗室への移行率*	反応潜時の中央値
対照（緩衝液投与）	０／１０^{aA}	300秒以上
スコポラミン(Sc)単独	９／１０^{bB}	128秒
Sc＋ウシ脳ＰＳ	０／１０^{A}	300秒以上
Sc＋ブタ脳ＰＳ	０／１０^{A}	300秒以上
Sc＋ヒツジ脳ＰＳ	０／１０^{A}	300秒以上
Sc＋鶏肉ＰＳ	０／１０^{A}	300秒以上
Sc＋鶏肝臓ＰＳ	０／１０^{A}	300秒以上
Sc＋イワシ魚肉ＰＳ	０／１０^{A}	300秒以上
Sc＋サバ血合肉ＰＳ	０／１０^{A}	300秒以上
Sc＋綿実ＰＳ	０／１０^{A}	300秒以上
Sc＋酵母ＰＳ(S.cerebisiae)	０／１０^{A}	300秒以上
Sc＋合成ＰＳ(Dioleoyl)	０／１０^{A}	300秒以上
Sc＋合成ＰＳ(Dipalmitoil)	０／１０^{A}	300秒以上

＊：５分以内に暗室に移行した動物数／全動物数
a vs b：p<0.01（マン・ホイットニーのU検定）
A vs B：p<0.01（ノンパラメトリック多重比較）

【００３４】表２に示すように、対象群（緩衝液を投与）では、全ての個体の反応潜時は、５分以上であったのに対し、スコポラミン単独投与群では１０頭中９頭の個体が５分以内に暗室に入り、記憶障害の誘発が確認された。一方、スコポラミンとＰＳとを両方投与したものでは全ての個体が暗室に留まっており、ウシ脳以外から抽出したＰＳもウシ脳ＰＳと同様に抗痴呆効果（スコポラミン誘発記憶障害の改善効果）を持つことが確認された。尚、同様の抗痴呆効果はこれらのＰＳのリゾ体でも確認された。

【００３５】以上のように、ウシ脳から抽出されたホスファチジルーＬーセリン以外でも、脳機能改善効果があるとの本発明の知見により、

(1) 脳機能改善に有効なホスファチジルーＬーセリンを経口で摂取できるため、苦痛なく容易に連続摂取が可能となった。

(2) 安価に入手できる原料材料から脳機能改善に有効なホスファチジルーＬーセリンを安価に、大量に製造できるようになった。

【００３６】

【発明の効果】本発明は以上説明したとおり、ウシ以外の動物の脳由来のホスファチジルーＬーセリン又はその塩を有効成分とするものであるため、脳内グルコース濃度を増加させることができる。従って、投与された被験体の脳機能を改善する効果を有する。また、具体的なウシ以外の動物としては、豚、羊、鶏等が上げられる。

【００３７】また別の本発明の脳機能改善剤では、綿実由来のホスファチジルーＬーセリン又はその塩を有効成分とするもの、酵母由来のホスファチジルーＬーセリン又はその塩を有効成分とするもの、更に、鶏肉，鶏内臓，魚体，魚肉の何れか由来のホスファチジルーＬーセリン又はその塩を有効成分とするものであるため、同じく、脳内グルコース濃度を増加させることができる。従って、投与された被験体の脳機能を改善する効果を有する。

【００３８】以上のように、本発明に係る脳機能改善剤では、ウシ脳以外のホスファチジルーＬーセリンは、何れもウシ脳に比べてはるかに大量にまた安価に提供可能であり、また供給面での量的問題も少ない。

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APPLICANT : YAKULT HONSHA CO LTD;

INVENTOR : OWAYA HIDEYUKI;

INT.CL. : A61K 31/66

TITLE : AGENT FOR IMPROVING CEREBRAL FUNCTION

ABSTRACT : PURPOSE: To obtain a cerebral function improving agent containing phosphatidyl-L-serine originated from the brain of an animal except a cattle, capable of being massively produced at a low cost, without a problem on the aspects of the cost and supply, and capable of improving the cerebral functions.

CONSTITUTION: This improving agent contains phosphatidyl-L-serine originated from the brain of an animal (e.g. pig, sheep, chicken) except cattle or its salt as an active ingredient. The salt of the phosphatidyl-L-serine is preferably Na salt or K salt. The phosphatidyl-L-serine is obtained e.g. by extracting a swine brain or an ovine brain with acetone, filtering the filtrate, extracting the residue with petroleum ether, vacuum-drying the extract, dissolving the dried product in ethyl ether, adding ethyl alcohol to the solution to produce precipitates, dissolving the precipitates in chloroform, and subsequently purifying the solution with silica gel column chromatography.

⑲日本国特許庁　　⑪特許出願公開

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（全 3 頁）

⑤4魚類餌料の製造法

⑦2発明者　柴田精三
市川市国分2—16—9
⑦1出願人　日本ペイント株式会社
大阪市大淀区大淀北2丁目1番2号
⑦4代理人　弁理士　青山葆　外1名

②1特願　昭52—26774
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⑦2発明者　伊奈和夫
静岡市柚木春日丘591—3

明　細　書

1.発明の名称

魚類餌料の製造法

2.特許請求の範囲

1.魚類の内臓および／または頭部に、低級アルコールを除く有機溶剤を添加混合して脱脂、脱水および脱臭を行ない、乾燥した後粉砕し、次いで得られる粉末に植物性蛋白質および／または微生物蛋白質を調合することを特徴とする魚類餌料の製造法。

2.有機溶剤が石油系溶剤、エーテル類、エステル類、芳香族系溶剤、ケトン類および炭素数5以上のアルコール類の群から選ばれる少なくとも1種である上記第1項記載の方法。

3.有機溶剤がアセトンである上記第1項記載の方法。

4.a.魚類の内臓および／または頭部に、低級アルコールを除く有機溶剤を添加混合して脱脂、脱水および脱臭を行ない、乾燥した後粉砕して得られる粉末、およびb.植物性蛋白質および／または微生物蛋白質から成ることを特徴とする魚類餌料。

5.粘着剤、消化酵素および栄養剤の群から選ばれる少なくとも1種をも含有する上記第4項記載の物。

3.発明の詳細な説明

本発明は魚類餌料の製造法、更に詳しくは、水産加工により廃出する魚類（海水、淡水中に棲息する全ての動物を指称する）の内臓や頭部を原料とし、これに有機溶剤を作用させて脱脂、脱水および脱臭を行ない、乾燥後粉砕化して得られる粉末を、魚類餌料にほとんど利用されていない植物性蛋白質や微生物蛋白質と調合することから成る、新規魚類餌料の製造法に関する。

従来より、例えば魚類の内臓を加工処理して、直接餌料にもしくはその基材として利用する試みがなされている。しかし、この場合加工時の強烈な魚臭は免がれず、しかも加工品は魚油の酸敗を招くことが多く、これらの公害基因により製造中止の現状にあつた。

そこで、本発明者らは、このような環境汚染物

質である魚類廃棄物の有効な加工処理について鋭意研究を進めた結果、低級アルコールを除く有機溶剤で処理すれば有効に脱脂、脱水および脱臭が行なわれ、上記悪臭の発散を解消することができ、しかもかかる処理物は簡単な乾燥、粉砕処理で効率よく魚類（食用に供される全ての魚種を指称する）に対して摂餌能力を有する粉末状の動物性蛋白質とすることができることを見出した。更に、この粉末に一般に魚類餌料としてほとんど利用されていなかつた植物性蛋白質や微生物蛋白質を調合することで、これらの蛋白質の有効利用が可能であることを見出した。

本発明は、上述の知見に基いて完成されたもので、その要旨は、魚類の内臓および／または頭部に、低級アルコールを除く有機溶剤を添加混合して脱脂、脱水および脱臭を行ない、乾燥した後粉砕し、次いで得られる粉末に植物性蛋白質および／または微生物蛋白質を調合することを特徴とする魚類餌料の製造法に存する。

本発明に使用する原料としては、魚類の廃棄物が採用されてよく、例えば水産加工により廃出される内臓および頭部、更に生鮮魚類処理により得られるその他の廃棄物が挙げられる。かかる原料は、生ものまたは乾燥物のいずれであつてもよく、また磨砕あるいは粉砕して使用に供することが好ましい。

本発明に使用する有機溶剤としては、低級アルコール以外のものであればいずれであつてもよく、例えば石油系溶剤（石油エーテルなど）、エーテル類（エチルエーテルなど）、エステル類（酢酸エチルなど）、芳香族系溶剤（キシレンなど）、ケトン類（アセトンなど）および炭素数５以上のアルコール類（ｎ－ヘキサノールなど）が挙げられ、これらの群から選ばれる少なくとも１種を使用に供する。中でも、アセトンの使用が好適である。

以下、本発明方法の手順について詳述する。

先ず、上記原料に有機溶剤を添加し、常温にて攪拌混合して脱脂、脱水および脱臭を行なう。

次に、かかる処理された原料を、通常６０℃以下、好ましくは４０～６０℃の温度で乾燥する。乾燥温度が６０℃を越えると、変質する傾向にある。乾燥後、常法に従つて粉砕処理に付す。このようにして、魚類餌料に好適な動物性蛋白質の給源である原料粉末が得られる。

この粉末には、魚類の嗜好性物質が含まれるため、これに通常の植物性蛋白質や微生物蛋白質（以下、添加蛋白質と称す）を添加し、常法に従い調合することにより目的とする魚類餌料が製造される。なお、かかる原料粉末と添加蛋白質の他の調合法として、上記溶剤処理後の未乾燥原料に当該添加蛋白質を添加し、これらを先に粉砕してから乾燥する方法が採用されてもよい。

以上の如くして製造される、本発明の魚類餌料にあつては、通常の結着剤（α－澱粉、小麦粉など）、消化酵素（蛋白質分解酵素、セルロースまたは澱粉分解酵素など）、栄養剤（メチオニン、アラニン、リジン等のアミノ酸、ビタミン類、ビタミン関連物質など）等が適量配合されてもよい。

以上の構成から成る本発明方法によれば、従来より腐敗と悪臭のため利用し得なかつた、魚類廃棄物の動物性蛋白質を脱脂、脱水および脱臭することにより、これを有益な飼料原料とすることができ、そしてこれに通常の植物性蛋白質や微生物蛋白質を調合することにより、これらの利用範囲を拡大することが可能となる。

次に、実施例および試験例を挙げて本発明を具体的に説明する。なお、実施例１～６は添加蛋白質を含まない参考例である。

実施例１

生マグロの内臓１０㎏に等量（Ｗ／Ｖ）のアセトンを加え、充分攪拌して脱脂、脱臭、脱水をする。固形物とアセトン溶液を分離後、更に固形物を１／３容量（Ｗ／Ｖ）の上記アセトン溶液で洗浄する。次いで、固形物からアセトンを回収すると同時に乾燥し、続いて粉砕機にかけて粉末とする。かかる粉末に少量のビタミン類および小麦粉約１㎏を混ぜて、魚類餌料を製造する。

実施例２

生カツオの内臓１０㎏に等量（Ｗ／Ｖ）のアセ

トンを加え、以後実施例1と同様に処理して、魚類餌料を製造する。

実施例3

生サンマの内臓10Kgに等量（W／V）のアセトンを加え、以後実施例1と同様に処理して、魚類餌料を製造する。

実施例4

生イワシの内臓10Kgに等量（W／V）のアセトンを加え、以後実施例1と同様に処理して、魚類餌料を製造する。

実施例5

生アジの内臓10Kgに等量（W／V）のアセトンを加え、以後実施例1と同様に処理して、魚類餌料を製造する。

実施例6

生タラの内臓10Kgに等量（W／V）のアセトンを加え、以後実施例1と同様に処理して、魚類餌料を製造する。

実施例7

実施例1の餌料5Kgに、植物性蛋白質（不二製油社製商品名「フジプロSR」、分離蛋白質）5Kgを混合し、魚類餌料とする。

実施例8

実施例2の餌料5Kgを、実施例7に準じて魚類餌料とする。

実施例9

実施例3の餌料5Kgを、実施例7に準じて魚類餌料とする。

試験例1

試験水槽

海水魚（タイ、ハマチ、アジ）や淡水魚（コイ、アユ、マス）の遊泳魚については、直径150cm、深さ13.0cmの水槽に水位100cmとし、ウナギ、ナマズなどの比較的動きのにぶい魚については、縦125cm，横100cm，深さ25cmの水槽を用いて試験を行つた。

供試魚

供試魚として各魚種について孵化後の日数はまちまちであるが、体長5～10cm程度の比較的稚魚を用い、維持餌料としては市販配合餌料を用いた。

造肉試験

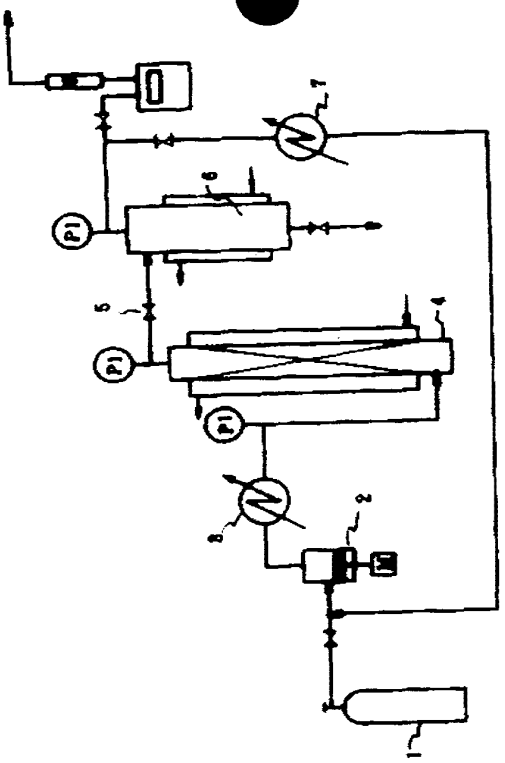
前記試験水槽を各魚種について2個宛用意し、一方を試験区、他方を対照区とし、試験区および対照区共15匹の供試魚を放養した。試験区には、各実施例で製造した餌料を魚体重と餌料蛋白量との関係より求め給餌した。対照区は、市販各魚種に対する餌料を蛋白質量において試験区と同一になるよう給餌し、3ケ月間試験給餌した後各区の魚を取り上げ試験前と試験後の体重を測定し、対照区との体重の比較をもつて餌料効率を求めた。その結果を第1表に示す（なお、対照区の体重増加を1.0として、試験区の体重増加をその比で表わす）。

第1表

供試魚 ＼ 試験		実施例 1	2	3	4	5	6	7	8	9
マダイ	判定	+	+	+	+	+	+	+	+	±
	飼料効率	1.1	1.0	1.1	1.0	1.1	1.2	1.1	1.0	0.9
ハマチ	判定	+	+	+	+	−	+	−	+	+
	飼料効率	1.1	1.0	1.1	1.0	1.2	1.0	1.1	1.0	1.0
アジ	判定	+	+	+	+	+	+	−	+	+
	飼料効率	1.2	1.1	1.0	1.1	1.0	1.2	1.0	1.0	1.1
ウナギ	判定	+	+	+	+	+	+	+	+	±
	飼料効率	1.0	1.0	1.1	1.0	1.2	1.1	1.1	1.0	0.9
アユ	判定	+	+	+	−	+	+	+	+	+
	飼料効率	1.2	1.1	1.2	1.0	1.0	1.0	1.0	1.2	1.0
マス	判定	−	+	+	+	+	+	+	−	+
	飼料効率	1.1	1.0	1.1	1.2	1.0	1.1	1.1	1.0	1.0
コイ	判定	+	+	+	+	+	+	+	+	+
	飼料効率	1.0	1.2	1.1	1.1	1.1	1.2	1.0	1.0	1.0
ナマズ	判定	+	+	+	+	+	+	+	+	+
	飼料効率	1.1	1.2	1.1	1.0	1.2	1.1	1.0	1.0	1.0

EUROPEAN PATENT OFFICE

Patent Abstracts of Japan



PUBLICATION NUMBER : 60035057
PUBLICATION DATE : 22-02-85

APPLICATION DATE : 27-06-84
APPLICATION NUMBER : 59133958

APPLICANT : SAN EI CHEM IND LTD;

INVENTOR : KOTAKE KINNOSUKE;

INT.CL. : C09B 61/00

TITLE : PRODUCTION OF YELLOWISH ORANGE TO REDDISH ORANGE DYE

ABSTRACT : PURPOSE: To obtain the titled dye which has excellent light resistance and color-developability and does not emit malodor, by neutralizing a solvent extract of krill, adding lipase or an alkali thereto to decompose fatty acids and impurities and extracting the treated soln. with a fluid in a super critical state.

CONSTITUTION: Krill are extracted with an org. solvent such as acetone or n- hexane. 0.1~1pt.wt. water is added to 1pt.wt. crude dye soln. obtd. by neutralizing the pH of the extract. 50~200 unit of lipase is added thereto and the mixture is heated at 35~40°C for 10~40hr. Alternatively, 0.1~5pts.wt. alkali and 5pts.wt. ethyl alcohol are added to the mixture of water and the extract, the resulting mixture is refluxed for 1~5hr and the pH thereof is then adjusted to 3 or below, whereby fatty acids and impurities are decomposed. The treated soln. is fed to an extraction tower 4 and a fluid (e.g. CO_2) brought into a super critical state by a compressor 2 and a heat exchanger 3 is introduced into the tower 4 where said treated soln. is extracted with said fluid at 30~90°C under a pressure of 70~500kg/cm^2. The pressure of the fluid is reduced by passing it through a reducing valve 5, fatty acids, etc. are separated in a separator 6 and the fluid is passed through a condenser 7 and recycled to the compressor 2.

⑲日本国特許庁（JP）　⑪特許出願公開

⑫公開特許公報（A）　昭60－35057

⑤1Int.Cl.⁴　識別記号　庁内整理番号　⑬公開　昭和60年(1985)2月22日
C 09 B 61/00　Z－6464－4H

審査請求　未請求　発明の数 1　（全4頁）

⑤4発明の名称　黄橙色ないし赤橙色色素の製法

⑳特　願　昭59－133958
㉒出　願　昭58(1983)6月23日
㉜特　願　昭58－114027の分割

⑦2発明者　安田　侃　豊中市千里園1丁目9番29号
⑦2発明者　小竹　欣之輔　豊中市走井1丁目18番6号
⑦1出願人　三栄化学工業株式会社　豊中市三和町1丁目1番11号

明　細　書

1. 発明の名称

黄橙色ないし赤橙色色素の製法

2. 特許請求の範囲

(1) オキアミの溶剤抽出液より黄橙色ないし赤橙色色素を抽出分離するに当り、該溶剤抽出液をそのpHを中性にした後、リパーゼあるいはアルカリを添加して、脂肪酸その他の夾雑物を分解して液系とし、これに抽剤として超臨界状態の流体を用いることを特徴とする黄橙色ないし赤橙色色素の製法。

(2) 超臨界状態の流体が二酸化炭素である特許請求の範囲(1)記載の方法。

(3) 超臨界状態の流体の圧力70～300㎏／㎠、温度30～60℃の範囲で抽出を行なう特許請求の範囲(1)または(2)記載の方法。

3. 発明の詳細な説明

この発明は色素の製法に係るものであり、優れた品質の黄橙色ないし赤橙色色素を工業的に有利に取得する方法に関するものである。

従来オキアミ（Euphausia superba）は体内に黄橙色ないし赤橙色色素たるアスタキサンチンを含有する生物であること、多量に漁獲できること、天然物であるので無害であることなどの理由から色素原料として用いられているが、その市販色素には下記のごとき多くの欠点がある。

(1)特有の異臭を伴うこと、②発色が良くないこと、③光により退色しやすいことなどである。

本発明者はこの様な状況に鑑みオキアミから前記色素を製造する方法について研究の結果、次の知見を得た。即ちオキアミ色素中に介在ないしは結合する不飽和脂肪酸やそのグリセリンエステルその他脂質が酸化分解するため前記の異臭を放つ要因となること、またその過程で生ずる反応生成物が色素の退色の一因となっていること、さらにはそれらの脂質と色素が効率良く分離できないため、低濃度の色素しか得られず、従って発色不良の原因となっている。本発明は、これらの知見にもとづいて創出されたものである。即ち本発明の目的はオキアミの溶剤抽出液より高品位の黄橙色ないし

赤橙色色素を工業的に有利に抽出分離する方法を提供するにあり、その要旨はオキアミの溶剤抽出後をそのｐＨを中性にした後リパーゼあるいはアルカリを添加して脂肪質その他の夾雑物を分解して液系とし（以下脂質分解処理液という）これに超臨界状態の流体を抽剤として抽出分離することを特徴とする黄橙色ないし赤橙色色素の製法である。

超臨界状態の流体を用いて有機化合物をそれを含有する混合物から分離する方法は例えば特公昭５４－１０５３９号公報に記載のとおり公知であるがオキアミより色素を分離する方法については未だ全く知られていない。

超臨界状態の流体とは臨界温度および臨界圧力を超える状態にある流体であって例えばエチレン（９．９℃、５０．５ａｔｍ）、アンモニア（１３２．３℃、１１１．３ａｔｍ）二酸化炭素（３１．０℃、７２．９ａｔｍ）のごとくの臨界状態以上の条件にある流体であって液体に近い密度とガス体に近い大きな拡散係数を有する流体である。この物性の故に種々

の特定の化合物を速かにかつ大量に効率よく抽出でき、しかも製品に抽剤が残らないというすぐれた特徴を有する。本発明に於いては一般に前記超臨界にある流体のいずれもが使用可能であるが、対象が食品に関するものであるため二酸化炭素を用いると無害かつ不活性であり、比較的低温での処理が可能で取扱いおよび操作が簡単で経済的に有利であるなど数々の利点を有するので通常抽剤として二酸化炭素を用いる場合が多い。

原料にはオキアミの生体またはそれらの乾燥体を溶剤で浸漬し、色素を溶出した後、その溶剤を留去させた粗色素液を用いる。溶剤にはアセトン、ｎ－ヘキサン、酢酸エチル等の有機溶剤が用いられる。

脂質の除去は、脂質の分解と、その除去の２工程により達成される。脂質の分解には、リパーゼを用いた酵素的な方法と、アルカリ剤を用いた化学的な方法がある。

分解後のグリセリン、脂肪酸の除去には超臨界状態の流体を抽剤として用いる。

まず酵素による分解であるが、粗色素液１部に対し要すれば約０．１～１部（重量、以下同じ）の水を加えた後、ｐＨ７．０に調整し少量の水に溶解したリパーゼを、粗色素液１ℓ当たり５０～２００ｕｎｉｔ（国際単位、以下同じ）加え、約３５～４０℃で１０～４０時間攪拌する。次いで約８０～１００℃に加温し、酵素を失活させると共に静置して、上部の色素油層を分離する。

アルカリによる脂質の分解は、色素液１部に約０．１～０．５部の水酸化カリウムその他のアルカリ質と、約５部のエチルアルコールを加え攪拌下に１～５時間還流した後、希硫酸水でｐＨ３．０以下にし、常法により上部の色素液層を分離する。

次いで分離した脂肪酸グリセリンおよびその他の脂質の除去を抽剤として超臨界状態の流体を用いて行なうが、以下に本発明の実施態様をフローシートに基づいて説明する。

第１図において二酸化炭素シリンダー１より圧縮機２を用いて所定の圧力まで圧縮した二酸化炭素を熱交換器３を通して所定の抽出温度にし超臨

界状態にして抽出塔４へ導入する。抽出塔４には上記オキアミの溶剤抽出液の脂質分解処理液を仕込んでおき、ここで脂肪酸、グリセリン等を抽出した二酸化炭素相を減圧弁５を通して減圧し、セパレーター６に導き脂肪酸およびグリセリン等を二酸化炭素から分離する。減圧された二酸化炭素はコンデンサー７で冷却液化し圧縮機２を経てリサイクルする。

上記プロセスにおいて抽出塔内の超臨界状態の二酸化炭素の圧力は７０～５００㎏／㎠、好ましくは７０～３００㎏／㎠、温度は３０～９０℃好ましくは３０～６０℃の範囲に保って抽出することが必要である。低過ぎると超臨界状態とならず高すぎると装置費が嵩むばかりか熱劣化などの悪影響の現われる場合もある。抽剤が二酸化炭素の場合は特に圧力２００㎏／㎠前後温度４０℃前後の条件を用いると好結果が得られる。

本発明の方法により、主として色素以外の油成分が抽出除去され抽出残として色素濃縮物を得ることができる。

このようにして得られた色素は本発明の目的とする高濃度で発色がよく特異臭も除去できた比較的安定性の優れた高品位のオキアミ色素であった。この色素を要すれば再び無臭な油脂で濃度調整または親水性の界面活性剤等によって乳化し水分散タイプに調整して目的とするオキアミ色素を得る。

このオキアミ色を食品に添加することによって鮮明な黄橙色ないし赤橙色が得られる。その食品の風味に対してこの色素は影響を与えないものである。

以下に本発明の効果の試験結果を第1表に示す。

第1表

（オキアミ色素の変化）

色素液	(A) 原液 オキアミの溶剤抽出液	(B)リパーゼによる脂質分解後濃縮した液	(C)アルカリ質による脂質分解後濃縮した液
含量（ppm）	199	2838	1780
色価 10% (E/n-Hexane)	46.0	640.5	411.5

色素含量は（2338 ppm）であった。さらにこの色素濃縮抽出残を（バタークリーム）に（0.013%）添加着色したところ未精製品を使用した場合と比べて異臭もなく鮮明な橙色に着色された。

実施例2

実施例1で用いたと同じオキアミ色素液100gを95%エチルアルコール500gに苛性カリ30gを溶解した液に加え攪拌下にN₂ガスを吹き込みながら約2時間還流し中性脂質を分解した。その後希硫酸でPH 2.5に調整し、多量の塩水を加えた後ジエチルエーテルにて常法により色素油分を抽出した後エーテルを留去して色素液を得た。この色素液に1ℓの抽出塔に仕込み第1図に示すプロセスにより抽出温度45℃、圧力200 kg/cm²の二酸化炭素を用いて抽出精製し常温大気圧下で抽出物の分離を行ない10.3gの色素濃縮抽出残を得た。この色素濃縮抽出残の色素含量は1780 ppmを抽出前色素液と比べて臭気も少なかった。この色素濃縮抽出残を用いえびせんべいに0.11%で着色したところ非常に鮮明に橙色に着色できた。

（着色剤の着色濃度）

色素液	(A) 原液	(B)	(C)
かまぼこ	2	0.17	0.22
マヨネーズ	0.2	0.015	0.02
バタークリーム	0.15	0.013	0.017
せんべい	1	0.08	0.11
あめ	1	0.08	0.11
チューインガム	0.1	0.08	0.01

実施例1

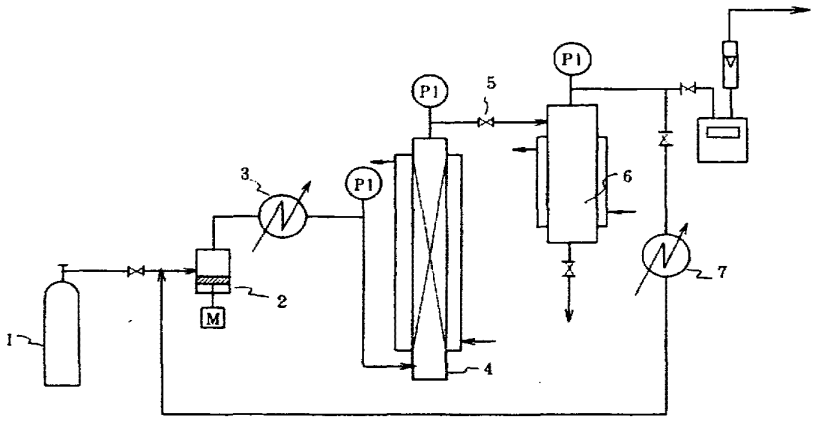
オキアミ色素液（色素含量199 ppm）100gに水100 mlを加えた液を希硫酸でPH 7.0に調整し、次いでリパーゼ（30,000 unit/g）0.5gを50 mlの水に溶かした酵素液を添加した後38℃にて25時間攪拌した。次いで90℃で30分間加熱後静置して上層の色素を分離した。この分離した色素液を内容積1ℓの抽出塔に仕込み第1図に示すプロセスにより抽出温度45℃、圧力200 kg/cm²の二酸化炭素を用いて抽出精製し、常温大気圧下で抽出物の分離を行ない（7.6g）の色素濃縮抽出残を得た。この色素濃縮抽出残の

異味異臭は感じられなかった。

4.図面の簡単な説明

第1図は本発明の実施態様を示すフローシートである。

特許出願人
三栄化学工業株式会社



PUBLICATION NUMBER : 04057853
PUBLICATION DATE : 25-02-92

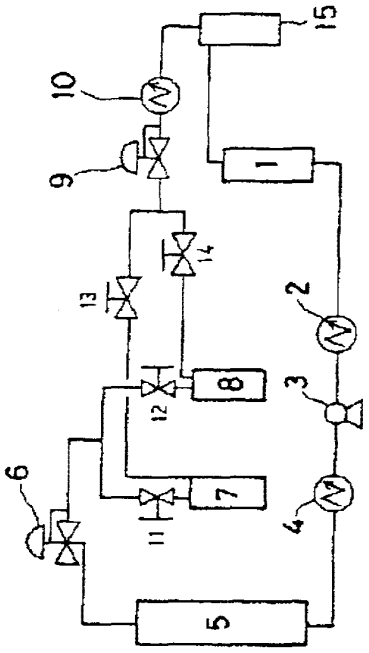
APPLICATION DATE : 28-06-90
APPLICATION NUMBER : 02170549

APPLICANT : ITANO REITOU KK;

INVENTOR : TANI TOSHIFUMI;

INT.CL. : C09B 61/00

TITLE : METHOD FOR EXTRACTING AND SEPARATING COLORING MATTER FROM KRILL



ABSTRACT : PURPOSE: To prepare a reddish orange coloring matter having a high safety in a high concn. by extracting, with CO_2 in a supercritical state, krill shells of which the protein has been decomposed by a protease.

CONSTITUTION: Krill shells are treated with a protease to decompose the protein in the shells and the treatment product is filtered. The residue of filtration is dried to give treated shells having a water content of 6-8% and a mean particle size of 200 μm or lower. The treated shells are put into an extraction vessel 5. An extractant comprising a liq. CO_2 in an amt. of 30-40 pts.wt. based on one pt.wt. treated shells having a coloring matter concn. of 30 mg/100 g is supplied through a supercooling apparatus 2 to a pump 3, pressurized at the pump 3 to 100-250 kg/cm^2, heated with a heat exchanger 4 to 35-40°C to bring it into a supercritical state, and transferred to the extraction vessel 5 to extract an oil in the treated shells. After the pressure of the oil-contg. CO_2 in the supercritical state is reduced to 40-60 kg/cm^2 with a pressure reducing valve 6, the CO_2 is delivered through a selector valve 11 to the first separating vessel 7 to separate the oil, and recycled through a selector valve 13, a pressure reducing valve 9, a condense 10, a water separator 15, and a storage vessel 1 to the extraction vessel 5. Then, selector valves 11 and 13 are closed while selector valves 12 and 14 are opened, and the CO_2 contg. the coloring matter is transferred to the second separating vessel 8, where the CO_2 is evaporated to give a coloring matter with a concn. of 2000-10000 mg/100g.

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　平4−57853

⑤Int.Cl.[5]	識別記号	庁内整理番号	㊸公開　平成4年(1992)2月25日
C 09 B 61/00	A	7537−4H	

審査請求　未請求　請求項の数 5　(全7頁)

㊹発明の名称　オキアミからの色素の抽出分離方法

㉑特　願　平2−170549
㉒出　願　平2(1990)6月28日

⑫発明者	徳森	恒雄	岡山県倉敷市茶屋町2097−3　コーポラスサン201号
⑫発明者	隅田	葉子	岡山県岡山市学南町1−6−29
⑫発明者	津山	宏一	岡山県岡山市新保1135−10　セジュール新保北202
⑫発明者	国代	佳予子	岡山県岡山市新保706−1
⑫発明者	岡田	治男	徳島県麻植郡鴨島町牛島1068番地
⑫発明者	谷	敏文	徳島県鳴門市北灘町粟田字東傍示26−4
⑪出願人	クロリンエンジニアズ株式会社		東京都港区虎ノ門2丁目1番1号　商船三井ビル
⑪出願人	イタノ冷凍株式会社		徳島県鳴門市瀬戸町明神字弐軒家33番地の2
⑭代理人	弁理士 米澤	明	外7名

明細書

1. 発明の名称

オキアミからの色素の抽出分離方法

2. 特許請求の範囲

(1) オキアミが含有する色素を抽出分離する方法において、オキアミをタンパク分解酵素によって分解してタンパク質を除去した残渣であるオキアミ殻を原料として、超臨界状態の二酸化炭素を抽出剤として色素を抽出分離することを特徴とするオキアミからの色素の抽出分離方法。

(2) 抽出分離が超臨界状態の二酸化炭素の圧力を2段階に変えて、オキアミ殻の抽出物を分画することを特徴とする請求項1記載のオキアミからの色素の抽出分離方法。

(3) 抽出分離が超臨界状態の二酸化炭素の圧力を変えずに抽出物を経時的に分離することによって分画することを特徴とする請求項1記載のオキアミからの色素の抽出分離方法。

(4) 抽出分離が抽出槽において抽出された成分を条件の異なる複数の分離槽によって分画することを特徴とする請求項1記載のオキアミからの色素の抽出分離方法。

(5) オキアミ殻の水分の含有率が10%ないし30%であることを特徴とする請求項1ないし4のいずれか1項に記載のオキアミからの色素の抽出分離方法。

3　発明の詳細な説明

[産業上の利用分野]

本発明はオキアミに含まれているアスタキサンチンを主成分とする赤橙色の色素を分離し高濃度の色素を得る方法に関し、とくに超臨界状態の二酸化炭素を用いて抽出分離する方法に関する。

[従来の技術]

オキアミに含まれているアスタキサンチンを主成分とする赤橙色の色素は、一般にオキアミ生体から有機溶剤を用いて抽出されている。この抽出物にはオキアミに含まれている脂肪類をはじめとして各種の成分が含まれている。とくに色素に介在あるいは結合している不飽和脂肪酸やそのグリセリンエステル等の酸化分解物が異臭を放ったり

あるいは不飽和脂肪酸等の酸化分解の過程での反応生成物が色素の退色の原因となるために色素抽出物中に含まれている色素のみを濃縮して分離することが必要である。

オキアミの色素の抽出液中から色素を濃縮して分離する方法として、例えば特開昭６０－４５５８号公報あるいは特公昭６１－５２１８３号には、オキアミをｎ－ヘキサン、アセトン等の有機溶剤で抽出したオキアミの色素の抽出液のｐＨを中性にした後、リパーゼによって脂質を分解するか、あるいはアルカリを添加して脂質あるいはその他の夾雑物を分解した液から、色素の液を分離し、さらにこの色素の液を分子蒸留域いは超臨界状態の流体によって抽出分離を行うことが提案されている。

［発明が解決しようとする課題］

従来提案されているオキアミ色素の濃縮分離方法では、オキアミの生体中から有機溶剤によるオキアミ色素の液の抽出工程、中和工程、リパーゼ又はアルカリによる脂質、夾雑物の分解工程、夾雑物の分解物とオキアミ色素の分離工程、分子蒸留又は超臨界状態の流体による抽出工程という数多くの工程が必要である。

また、オキアミに含まれる赤橙色色素はアスタキサンチンを主成分としてビタミンＥの１００ないし１０００倍もの抗酸化作用があることが報告されており、今後医薬品原料として利用されることが期待されているが、医薬品原料として用いる場合には、オキアミ色素の液の抽出工程において使用した有機溶剤の残留を完全に防ぐために脱溶媒等の工程が必要となる。

また、処理工程を経ていないオキアミを直接に超臨界状態の二酸化炭素等で抽出することも考えられるが、オキアミに含まれている大量の水分や各種の有用成分が同時に抽出されるために色素のみを濃縮して分離することは困難であった。

［課題を解決するための手段］

本発明者らは、上記のような課題を解決すべく鋭意研究を重ねた結果、本発明を想到した。

オキアミは、タンパク質などの有用な成分が多く含まれているので加工食品の原料等の用途に使用されている。これらの用途のなかにはオキアミをそのまま使用するのではなくオキアミ中に含まれているタンパク質を分離してアミノ酸原料などに利用することが行われているが、こうしたアミノ酸原料の採取の用途に利用されたタンパク質などを除去したオキアミの殻は、従来廃棄もしくは養殖魚の餌として利用されるのみであった。

本発明者らは、オキアミからタンパク質などの成分を採取した残りの殻を、色素の製造原料とすることによりオキアミから超臨界状態の流体を用いてオキアミの色素の液を抽出するという簡単な方法を採用することによって特別な前処理工程を経ることなくオキアミの色素を製造することをみいだしたものである。

すなわち、オキアミ中のタンパク質などの有用な成分を酵素によって分解した後に濾過等の方法によって得られる残渣であるオキアミ殻を原料として、超臨界状態の流体によって色素を抽出するものである。

本発明における超臨界状態の流体とは、臨界温度および臨界圧力を超過した状態の流体であって、二酸化炭素では３１℃以上、７５．３Ｋｇ／ｃｍ²以上、プロパンでは９６．７℃以上、４３．４Ｋｇ／ｃｍ²以上、エチレンでは９．９℃以上、５２．２Ｋｇ／ｃｍ²以上の状態にあるものを言う。これらの流体は、液体に近い密度と気体に近い大きな拡散定数を有していることを特徴としており各種の有機物の抽出分離に用いられている。とくに本発明の方法では、超臨界状態の流体として二酸化炭素を用いるものであるが、二酸化炭素を用いると、色素の抽出分離に必要な工程が簡略化されるばかりではなく、抽出剤として用いる二酸化炭素はたとえ抽出した色素中に残留しても危険性が全くなく、得られた色素は医薬品の分野をはじめとして多くの分野で問題なく利用することが可能となる。

さらに抽出剤として用いる超臨界状態の二酸化炭素は、炭化水素類のように空気中での爆発あるいは燃焼の危険がない。また、二酸化炭素は臨界温度および臨界圧力が比較的低いので、温度、圧

力を変化させることによって、溶解特性を変化させることを容易に行うことが可能であり、色素の抽出分離に適した溶解特性の抽出剤で抽出を行うことができる。

本発明の方法は、オキアミ殻を原料として超臨界状態の二酸化炭素で色素を抽出するものであるが、その際に効率的な色素の抽出分離を行うための抽出圧力、温度、分画方法等の抽出条件、原料のオキアミ殻の含水率を検討し、本発明の方法を完成させるに至った。

以下に図面を参照して本発明を説明する。

第１図は本発明の方法を実施する複数の分離槽の切換手段を有する装置のフローシートを示す。

抽出剤である二酸化炭素は液体二酸化炭素貯槽１から過冷却器２を通過した後にポンプ３で所定の圧力に加圧され、続いて熱交換器４において所定の温度に加温されて、超臨界状態の二酸化炭素としてオキアミ殻を充填した抽出槽５に供給される。

原料のオキアミ殻は、主としてキチン質、蛋白質、トリグリセライドエステル、ジグリセライドエステル、モノグリセライドエステル（油分）、色素（アスタキサンチン）を含むもので、凍結したオキアミを解凍後タンパク分解酵素によってタンパク質を分解したエキス分を濾過した際に得られる残渣で、平均粒径２００μｍの粉状体であり、乾燥したものは通常６％ないし８％含水率を有している。

超臨界状態の二酸化炭素はオキアミ殻の油分、色素を溶解する特性をもつためオキアミ殻からこの２成分のみが抽出されるが、これらの２成分を分取するために、抽出操作は２段階に分けて行われる。

すなわち、第１段の抽出は含有するアスタキサンチンの濃度に相当する色素濃度が３０ｍｇ／１００ｇのオキアミ殻１重量部に対して温度３５℃ないし５０℃で、圧力が相対的に低圧である圧力１００Ｋｇ／ｃｍ²ないし２５０Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を３０重量部ないし４０重量部を通じてオキアミ殻に含まれる油分を抽出する。

油分を含有する超臨界状態の二酸化炭素は、減圧弁６で圧力４０Ｋｇ／ｃｍ²ないし６０Ｋｇ／ｃｍ²まで減圧されて切換弁１１を通って第１分離槽７へ導かれる。

第１分離槽７内で油分を分離した気体状態の二酸化炭素は、切換弁１３および減圧弁９で更に減圧されて断熱膨張し、凝縮器１０で液化された後に水分離器１５を経て、液体二酸化炭素貯槽１へ戻されて循環使用される。

続いて、超臨界状態の二酸化炭素の圧力を第１段階の抽出時の圧力よりも相対的に高圧として抽出槽５に供給する。すなわち、抽出槽へ供給する超臨界状態の二酸化炭素を温度３０℃ないし５０℃、圧力を３００Ｋｇ／ｃｍ²ないし５００Ｋｇ／ｃｍ²として、オキアミ殻１重量部に対して３０重量部ないし４０重量部を通じ、切換弁１１および１３を閉じて、切換弁１２および１４を開いて減圧弁６により４０Ｋｇ／ｃｍ²ないし６０Ｋｇ／ｃｍ²とした抽出物を含む二酸化炭素は第２分離槽８へ導かれる。第２分離槽８において気体状態となった二酸化炭素は、第１段階の抽出工程と同様にして液体二酸化炭素貯槽１に戻され、第２分離槽８からは色素濃度が２，０００ｍｇ／１００ｇないし１０，０００ｍｇ／１００ｇという色素濃度の非常に大きな色素が得られる。

以上の様に低圧、高圧の２段階の抽出を連続的に行うことによって色素濃度の高い色素が得られるが、複数の分離槽を設けて、分離槽を切り換えることによって効率的な捕集が可能である。

また、上記のように圧力を変えて２段階の抽出を行わなくとも、同一圧力で同様の抽出分離を行うことも可能である。

すなわち、第２図に示すような抽出操作によっても高濃度の色素の抽出分離も可能である。第２図を参照して説明すると、液体二酸化炭素貯槽１から二酸化炭素は、過冷却器２を通ってポンプ３へ送られ、所定の圧力まで加圧される。続いて熱交換器４で所定の温度に加熱されて超臨界状態の流体とされ、あらかじめオキアミ殻を充填した抽出槽５へ供給される。

オキアミ殻1重量部（色素濃度30mg／100g）に対して、30重量部ないし50重量部の温度35℃ないし50℃、圧力300Kg／cm²ないし500Kg／cm²とした超臨界状態の二酸化炭素を通じる。抽出槽において抽出初期には油分が、その後は高濃度の色素が抽出され、抽出物を含んだ超臨界状態の二酸化炭素は、減圧弁6で圧力を40Kg／cm²ないし60Kg／cm²と減圧されて第1分離槽7へ導かれる。

第1分離槽から出た二酸化炭素は、減圧弁9でさらに減圧され、凝縮器10で液化された後、水分離器15を経て、液体二酸化炭素貯槽1へ戻される。

この抽出方法では、抽出の初期から比較的高圧の超臨界状態の二酸化炭素を使用するために、油分とともに色素も抽出され、色素の損失となるので、オキアミ殻1重量部に対して超臨界状態の二酸化炭素を15重量部ないし25重量部を供給して抽出を行った後に第1分離槽7の下部に設けた抜き出し弁16から油分を主とする抽出物が分離される。続いてオキアミ殻1重量部に対して超臨界状態の二酸化炭素を15重量部ないし25重量部を供給することにより、第1分離槽内に色素濃縮物が得られる。

本方法で得られる油分中の色素濃度は、10mg／100gないし30mg／100gであり、色素濃縮物中の色素濃度は、2,000mg／100gないし10,000mg／100gであった。

この方法では、抽出の初期から比較的高圧の超臨界状態の二酸化炭素を利用するために、若干の色素が初期抽出物である油分に含まれることになるが、前記の低圧、高圧の2段階の超臨界状態の二酸化炭素を用いる方法に比べて抽出時間の短縮が可能となるという特徴を有している。

また、第3図には連続的に複数の分離槽を設け、各分離槽の設定圧力および温度を変えることによって分離条件を変えて色素を効率よく回収できる方法を示す。

以下第3図を参照して説明する。

二酸化炭素は液体二酸化炭素貯槽1から、過冷却器2を通ってポンプ3へ送られて所定の圧力まで加圧される。その後、熱交換器4で所定の温度に加熱されて超臨界状態として、あらかじめオキアミ殻を充填した抽出槽5へ供給される。

ここで抽出槽5へ供給される超臨界状態の二酸化炭素は温度35℃ないし50℃、圧力300Kg／cm²ないし500Kg／cm²のものである。

抽出槽内でオキアミ殻より油分と色素が抽出され、この油分と色素を含む超臨界状態の二酸化炭素は減圧弁6で減圧され、高圧分離槽17へ導かれる。

高圧分離槽17は、抽出槽内より低圧の温度35℃ないし50℃、圧力100Kg／cm²ないし300Kg／cm²の超臨界状態に保たれており、槽内には色素濃縮物が捕集され、油分を含む超臨界状態の二酸化炭素は減圧弁18で減圧され低圧分離槽19に導かれる。

低圧分離槽17は温度20℃ないし30℃、圧力40Kg／cm²ないし60Kg／cm²ガス状態に保たれ、槽内では油分が捕集され、二酸化炭素は減圧弁9で更に減圧されて、凝縮器10で液化された後、水分離器15で含まれている水分を除去した後に液体二酸化炭素貯槽1へ戻される。

この方法を用いるとオキアミ殻1重量部（色素濃度30mg／100g）に対し比較的高圧の圧力300Kg／cm²ないし500Kg／cm²の超臨界状態の二酸化炭素を30重量部ないし40重量部通じることにより高圧分離槽に色素濃度が2,000mg／100gないし10,000mg／100gの色素を得ることができ、低圧分離槽には色素濃度の低い油分を抽出物として得ることができる。

抽出槽に供給する超臨界状態の二酸化炭素の圧力を当初は、300Kg／cm²より低圧の比較的低圧の超臨界状態の二酸化炭素を供給し、後に比較的高圧の超臨界状態の二酸化炭素を供給する方法は、初期抽出物中に色素はほとんど抽出されないので、色素の取得量は多くなるが、抽出に長時間を有し、一方、比較的高圧の300Kg／cm²ないし500Kg／cm²の超臨界状態の二酸化炭素を用いて抽出の当初は油分を抽出し、その後

に色素の濃縮物を抽出するという経時的に抽出物を分離する方法は、装置の構成が簡単であり抽出時間は短いものの初期抽出物として得られる油分中に色素がいくぶん含まれるために色素の取得量が減少するという問題点を有しているが、第３図に示す設定した圧力および温度が異なる複数の分離槽を設け、連続的に抽出条件の異なる成分の抽出物を得る方法は、前記の二者の方法よりも優れている。

また、本発明において原料として使用するオキアミ殻（色素濃度３０ｍｇ／100ｇ）は、オキアミ殻の水分の含有量を１０重量％ないし３０重量％と高めて抽出を行うことによって抽出速度、特に初期抽出速度を早めることが可能となるので、比較的高圧の３００Ｋｇ／ｃｍ²ないし５００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を用いた際の抽出の初期に得られる油分の抽出物中に含まれる色素の量を低下させることが可能となるので油分中に含まれて失われる色素の量の低下を防止することができる。

水を添加したために、水が色素とともに抽出されるが、水と色素は容易に２層分離することができるので、水を加えたことはなんら色素の抽出分離には障害とはならない。

しかしながら、含水率が３０％を越えると抽出初期の抽出速度の低下が見られるので水の量を３０％より多くすることは好ましくない。

オキアミ殻の含水率は、オキアミの処理工程での乾燥状態を制御することによって調整することが好ましいが、乾燥状態の比較的水分の含有量の小さいオキアミ殻を用いる場合には抽出工程の前にオキアミ殻に水を散布した後に十分に混和することが必要となる。

［作用］

本発明は、オキアミに含まれているアスタキサンチンからなる色素を製造する際に、オキアミからタンパク分解酵素によってタンパク質などの成分を採取した残りの殻を、色素の製造原料とし、超臨界状態の二酸化炭素を抽出剤として抽出したものであって、有機溶剤による特別な前処理工程を経ることなくオキアミの色素を製造することができる。

［実施例］

以下に本発明の実施例を示し、さらに詳細に本発明を説明する。

実施例１

タンパク分解酵素としてプロテアーゼを用い、凍結オキアミを解凍後４７℃ないし４８℃で２時間タンパク分解反応を行った後に、液体を濾過して分離した残渣を乾燥して含水率６％のオキアミ殻を得た。このオキアミ殻（色素濃度３０ｍｇ／100ｇ）６Ｋｇを容積２５リットルの抽出槽に充填し、抽出槽の温度を４０℃に保持し、温度４０℃、圧力２００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を１時間当り６０Ｋｇの供給速度で４時間供給し、圧力を５０Ｋｇ／ｃｍ²、温度３０℃とした分離槽において二酸化炭素と液体とを分離し、分離槽の下部の抜き出し弁から色素濃度７．１ｍｇ／100ｇの抽出物を１３９８ｇを得た。

さらに抽出槽中に温度４０℃、圧力４００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を１時間当り６０Ｋｇの供給速度で４時間供給し、分離槽の圧力を５０Ｋｇ／ｃｍ²、温度を３０℃として先の抽出の残渣から色素濃度８３３１ｍｇ／100ｇの高濃度の色素１３．４ｇを得た。

実施例２

実施例１で原料としたものと同一の成分のオキアミ殻（色素濃度３０ｍｇ／100ｇ）６Ｋｇを容積２５リットルの抽出槽に充填し、抽出槽の温度を４０℃に保持し、温度４０℃、圧力４００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を１時間当り６０Ｋｇの供給速度で２時間供給し、圧力を５０Ｋｇ／ｃｍ²、温度３０℃とした分離槽の下部の抜き出し弁から抽出物を取り出して色素濃度４２．８ｍｇ／100ｇの抽出物を１７０３ｇを得た。

さらに抽出槽中に引き続き温度４０℃、圧力４００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を１時間当り６０Ｋｇの供給速度で抽出開始から５時間供給し、圧力を５０Ｋｇ／ｃｍ²、温度３０℃とした分離槽において色素濃度５８７４ｍｇ／100ｇの

高濃度の色素１０ｇを得た。

実施例３

実施例１で原料としたものと同一の成分のオキアミ殻（色素濃度３０ｍｇ／100ｇ）２５０ｇを容積１リットルの抽出槽に充填し、抽出槽の温度を４０℃に保持し、温度４０℃、圧力４００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を１時間当り２．５Ｋｇの供給速度で２．５時間供給し、抽出槽で得られる抽出物を含む超臨界状態の二酸化炭素は超臨界状態に保持した高圧分離槽に供給した。

高圧分離槽は温度４０℃、圧力２５０Ｋｇ／ｃｍ²に保持し、低圧分離槽での抽出物は減圧弁を介して２０℃で６０Ｋｇ／ｃｍ²に保持した低圧分離槽に供給した。

この結果、高圧分離槽からは色素濃度７０７２ｍｇ／100ｇの色素を０．４４ｇ得ることができ、低圧分離槽からは色素濃度６．２ｍｇ／100ｇの色素を７０．５７ｇを得た。

実施例４

実施例１で使用したものと同一の成分のオキアミ殻６Ｋｇに水を散布した後に十分に混和させて原料の含水率を変化させて色素の抽出分離を行った。

容積２５リットルの抽出槽に含水率の異なるオキアミ殻を充填して抽出槽の温度を４０℃に保持し、温度４０℃、圧力４００Ｋｇ／ｃｍ²の超臨界状態の二酸化炭素を供給し、圧力を５０Ｋｇ／ｃｍ²、温度３０℃とした分離槽において分離し、抽出の開始時から得られた油分の分画物の量及び油分の分画が終了の後に得られた色素の量および抽出時間を表１に示す。

［以下余白］

表１

	油分		色素濃縮物	
含水率％	抽出時間（時）	抽出量（ｇ） 色素濃度（ｍｇ/100ｇ）	抽出時間（時）	抽出量（ｇ） 色素濃度（ｍｇ/100ｇ）
3	2	１５４９ １２．３	3	１８．７ １６６０
7	2	１７０３ ４２．８	3	６．８ ７８３２
14	1	１５４６ ２８．３	2	１１．９ ６０８４

［発明の効果］

本発明は、オキアミ殻からアスタキサンチンを含む赤橙色の色素を超臨界状態の二酸化炭素で抽出するものであり、従来オキアミから有用な成分を採取して廃棄物として取り扱われていたオキアミ殻を原料として有効に利用するものであり、しかも有機溶剤などを使用することがないので、有機溶剤の分離工程も必要でなく簡単な工程でかつ食品あるいは医薬品の分野においても安全性の高い色素を抽出分離することができる。

4．図面の簡単な説明

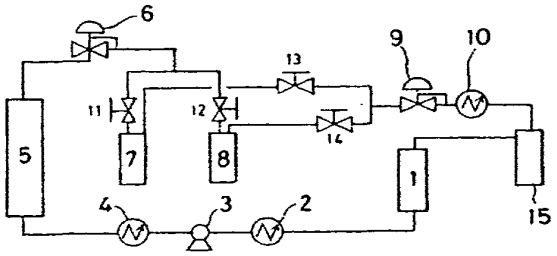
第１図は本発明の方法を実施するために使用する複数の分離槽を切り換える抽出装置のフローシートを示し、第２図は本発明の方法を実施するために同一の圧力で２種の成分を分画する際に使用する装置のフローシートを示し、第３図は本発明の方法を実施するために使用する圧力の異なる分離槽を有する装置のフローシートを示す。

液体二酸化炭素貯槽…１、過冷却器…２、ポンプ…３、熱交換器…４、抽出槽…５、減圧弁…６、第１分離槽…７、第２分離槽…８、減圧弁…９、凝縮器…１０、切換弁…１１、１２、１３、１４、水分離器…１５、抜き出し弁…１６、高圧分離槽…１７、減圧弁…１８、低圧分離槽…１９

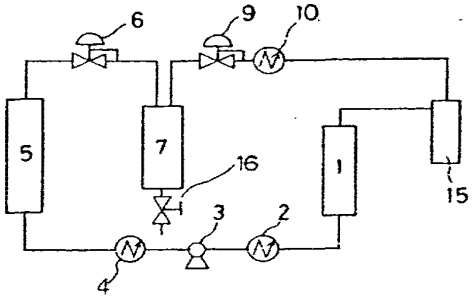
特許出願人　クロリンエンジニアズ株式会社
（外１名）

代理人　弁理士　米　原　　明　（外７名）

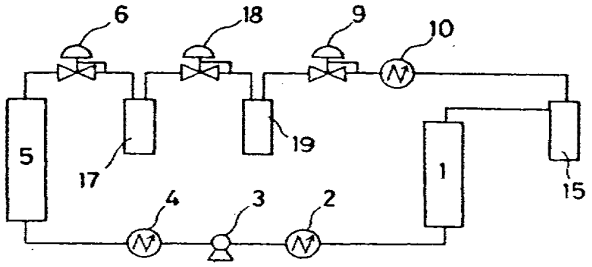
第 1 図



第 2 図



第 3 図



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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

5,094,679

(54) Process for Extracting Lipids with a High Proportion of Long Chain, Highly Unsaturated Fatty Acids

(72) Kohn, Gerhard - Germany (Federal Republic of) ;
Sawatzki, Günther - Germany (Federal Republic of) ;
Erbe, Jürgen - Germany (Federal Republic of) ;
Schweikhardt, Friedrich - Germany (Federal Republic of) ;

(71) Milupa Aktiengesellschaft - Germany (Federal Republic of) ;

(30) (DE) P 42 19 360.5 1992/06/12

(57) 12 Claims

Notice: This application is as filed and may therefore contain an incomplete specification.

ABSTRACT:

In the method of the invention to obtain lipids with a high proportion of long-chain polyunsaturated fatty acids (LCPs) with 20 to 22 carbon atoms by extraction from a raw material of animal or vegetable origin, unicellular algae (microalgae), macroalgae from the families of the brown, red and green algae and/or residues of alginate or carrageenin production with a water content of ≤ 50 weight % and a particle size of ≤ 50 mm are used. For extraction, an organic solvent or a compressed gas is used. A lipid extract with a high proportion of w6 LCP and w3 LCP and in particular with a content of at least 5 weight % of arachidonic acid and/or a content of at least 3 weight % of docosahexanoic acid is also prepared.

CLAIMS:

1. A method for obtaining lipids with a high proportion of long-chain polyunsaturated fatty acids (LCPs) having from 20 to 22 carbon atoms by extraction from a raw material of animal or vegetable origin,
characterized in that
as the raw material, unicellular algae (microalgae), macroalgae from the families of brown, red and green algae and/or residues from alginate or carragheenin production with a water content of $\leq$ 50 weight % and a particle size of $\leq$ 50 mm are used, and
that for extraction an organic solvent or a compressed gas is used.

2. The method of claim 1,
characterized in that
a raw material with a water content of 5 to 50% and in particular 5 to 15 weight % and with a particle size of 0.01 to 50 mm, in particular 0.1 to 1.0 mm, is used.

3. The method of claim 1 or 2,
characterized in that
extraction is done with a solvent miscible with water in any ratio, in particular a low alcohol, at a temperature of 20°C to 65°C, in particular 60°C, and that the extraction is carried out in the form of a batchwise extraction (maceration) percolation, decanter extraction or countercurrent extraction.

4. The method of one of claims 1-3,
characterized in that
the extraction liquid (miscella) obtained with the aid of a solvent miscible with water in any ratio at 20°C or more, in particular ethanol, is diluted with water, in particular to 20 to 90 volume %, and/or cooled down, in particular to < +20°C to -60°C, to such an extent that the lipid extract at least partly precipitates out, and
that the thus-obtained lipid extract is separated.

5. The method of one of the foregoing claims,
characterized in that
the miscella obtained with the aid of an organic solvent, or the extract obtained from it and partially or completely freed of solvent, is extracted with a compressed gas, and the neutral lipid fractions dissolved in the compressed gas and as desired the polar lipid fraction that has not entered into solution in the compressed gas are isolated.

6. The method of claim 1 or 5,
characterized in that
the pressures of the compressed gases employed are from 60 to 2000 bar, in particular 70 to 500 bar, and the temperature is from -20 to +200°C, in particular +20 to +60°C.

7. The method of claim 1, 5 or 6,
characterized in that
carbon dioxide is used as the compressed gas, to which an entraining agent may be added.

8. A lipid extract or lipid extract fraction of unicellular algae (microalgae), macroalgae from the families of the brown, red and green algae and/or the residues of alginate or carragheenin production,
characterized by
a content of arachidonic acid of at least 5 weight % and/or a content of docosahexanoic acid of at least 3 weight %, referred to the total weight of the fatty acids.

9. A lipid extract or lipid extract fraction, obtainable by the method of one of claims 1-7.

10. The lipid extract or lipid extract fraction of claim 9, characterized by a content of arachidonic acid of at least 5 weight % and/or a content of docosahexanoic acid of at least 3 weight %, referred to the total weight of the fatty acids.

11. A food, characterized in that it contains a lipid extract or a lipid extract fraction of one of claims 8-10.

12. The use of the lipid extract or lipid extract fraction of one of claims 8-10 as an additive to the fat body of infant nutrition products, as dietetic products for arteriosclerosis prevention, for the prevention of autoimmune diseases (atopias) and as an additive to cosmetic products.

PROCESS FOR EXTRACTING LIPIDS WITH A HIGH PROPORTION OF LONG-CHAIN HIGHLY UNSATURATED FATTY ACIDS

SPECIFICATION:

The invention relates to a method for obtaining lipids with a high proportion of long-chain highly unsaturated fatty acids, with from 20 to 22 carbon atoms, by extraction from a raw material of animal or vegetable origin, and to the extract obtained and its use.

Our foodstuffs include not only saturated fatty acids but also monounsaturated and polyunsaturated fatty acids, which thus have at least one double bond in their carbon chain. These polyunsaturated fatty acids are often designated by abbreviations. The number of carbon atoms or the chain length is given first. This is followed by a hyphen or colon, which in turn is followed by a number that indicates how many double bonds there are in the carbon chain. Following that but separately, the number of omega-carbon atoms is given, counted from the methyl end of the chain, after a "w" or "n". In this system, the short formula for linoleic acid is 18-2 n6.

In fatty acid metabolism in the human being, double bonds are known to be introduced into the carbon chain of a saturated fatty acid. However, this desaturation is possible only after the carbon atom C9 in the direction toward the carboxyl end. The result is that fatty acids such as linoleic acid (18:2 n6) and α-linolenic acid (18:3 n3) must be considered essential, since they cannot be synthesized by the human organism itself but rather must be supplied from food.

From these essential C18 fatty acids, the healthy human organism is capable of synthesizing a number of polyunsaturated fatty acids having from 20 to 22 carbon atoms, by means of further desaturation and chain elongation. The elongation occurs at the carboxyl end of the molecule, and the desaturation occurs between the carboxyl group and the first double bond that follows it. The number of carbon atoms between the methyl end of the fatty acid and the last double bond (omega-C atoms) remains unchanged thereby, so that from linoleic acid (18:2 n6) in lipid metabolism, only omega-6 fatty acids (w6 family) are derived, and from α-linolenic acid only omega-3 fatty acids (w3 family) are derived. The course of biosynthesis of the w6 family thus begins with linoleic acid (C18-2 n6) and proceeds through gamma-linolenic acid (C18-3 n6), di-homo-gamma-linolenic acid (C20-3 n6), and arachidonic acid (C20-4 n6) to docosapentanoic acid (C22-5 n6). With respect to the w3 family, the course of biosynthesis begins with α-linolenic acid (C18-3 n3), through octadecatetraenoic acid (C18-4 n3), eicosatetranoic acid (C20-4 n3), eicosapentanoic acid (C20-5 n3) to docosahexanoic acid (C22-6 n3).

By international convention, this group of fatty acids with extraordinary physiological importance is known as LCPs (for long-chain polyunsaturated fatty acids). These fatty acids with 20 to 22 C atoms are derived from the essential C18 fatty acids and have at least two double bonds in the acyl radical. The abbreviation LCP will be used below for this group of fatty acids, and a distinction is made between w6 LCPs and w3 LCPs.

The LCPs have versatile biological effects. For instance, they are an indispensable component of all the cell membranes in the body. A change in the membrane lipid composition can cause a great variety of physiological problems.

In recent years, the eicosanoids (prostaglandins, leukotrienes, prostacyclins and thromboxanes) synthesized in the organism from some LCPs have gained particular attention. It has been demonstrated that this highly active group of eicosanoids, in low concentrations, is involved in a number of physiological processes.

In infants and children, in comparison to adults, because of the relatively high need for growth and low reserves, the danger exists of a deficiency in these LCPs. In the last trimester of intrauterine fetal development and during postnatal development of the newborn, large amounts of w6 and w3 LCPs are accumulated in the organs. The capacity for synthesis of the LCPs from the essential precursors appears limited in the young infant, however, because of immaturity of the desaturating enzyme system.

Since these LCP fatty acids are virtually entirely absent from infant formulas previously available, formulas have recently been developed that are enriched with these fatty acids; see German Patent Disclosure DE 39 20 679 A1, for instance.

Because of the increased interest in LCPs, there has been an increased demand for sources of raw materials for such long-chain polyunsaturated fatty acids. The oils containing LCPs that are currently available are quite predominantly obtained from marine cold water fish (see European Patent Disclosure EP 0 292 846 A2 and German Patent Disclosure DE 39 40 239 A1). Such oils from the muscle tissue or organs of fish are distinguished by high proportions of w3 LCPs and in particular of eicosapentanoic acid (20-5 n3) and docosahexanoic acid (22-6 n3). Such oils and particularly oils from fish organs have the disadvantage, however, of a high cholesterol content and also a high content of fat-soluble vitamins and possibly fat-soluble pollutants (heavy metals and pesticides).

It has also already been proposed that LCPs be obtained from autotrophically or heterotrophically fermented microorganisms (see International Patent Disclosures WO 91/07498 and WO 91/119 182 and German Patent Disclosure DE 34 46 795 A1).

The LCPs of interest here can moreover be obtained from organ fats of livestock (cattle/pigs) and from the yolk of chicken eggs (EP 0 074 251 B2). The extraction of LCPs from human placentas is described in EP 0 140 805 A1.

The object of the present invention is to furnish a method for obtaining LCP-rich lipids from a raw material not previously used for these purposes. The object of the

invention is also to furnish a lipid extract or lipid extract fractions that are rich in LCP fatty acids and that provide a foundation for producing foods, in particular baby foods, among others.

This object is obtained by the teaching of the claims.

An essential aspect of the invention is that a certain raw material is used to obtain LCP-containing lipids.

In the method of the invention, one can for instance use macroalgae, primarily occurring in the sea, from the families of brown, red and green algae. Of these, those from the Phaeophyceae and Rhodophyceae families are of special interest. However, certain species are also used for human nutrition in other parts of the world, above all in the coastal countries of Northern Europe and East Asia (Japan). These macroalgae can be found in many continental shelf zones of the ocean and are available in practically unlimited quantities. A few macroalgae species are also intentionally cultivated in partitioned-off areas of the sea (aquaculture).

It has now surprisingly been found that lipids with a high proportion of LCPs can be extracted from these macroalgae in an economical way, if an organic solvent or a condensed gas is used. Moreover, the macroalgae are comminuted, in particular ground, before the actual extraction, so that the raw material obtained from these macroalgae and used in the method of the invention has a particle size of $\leq$ 50 mm. Furthermore, the macroalgae are dried either before or after the comminution, so that their water content amounts to $\leq$ 50 weight %.

Partially dried products of ground native algae available on the market ("algae flour") can also be used as raw material based on brown and red algae. These commercially available products are available and inexpensive on the market and were previously used only for soil improvement or as an additive to animal feed.

From various brown and red algae species, alginates and carragheenins are currently obtained on a large scale; they are used in the most versatile ways as hydrocolloids in the food industry. To obtain the hydrocolloids, the algae are "planted" or cultivated, harvested, dried and ground on a large scale as described above. Depending on the desired properties of the hydrocolloids to be extracted, different algae species are intentionally mixed and finally extracted in aqueous form.

It has also been surprisingly discovered that the residues that occur in the hydrocolloid extraction, which currently are not used at all or are used to only a very slight extent to produce products for soil improvement or as additives for animal feed, can be used as raw material for the method of the invention. It has in fact been demonstrated that in the extraction to obtain the hydrocolloids using acids and lyes, the LCP-containing lipids present in the algae are not damaged. On the contrary, by the removal of the hydrocolloids from the algae mixtures, there is in fact an improved yield of the extractable lipids, including the long-chain polyunsaturated fatty acids of interest here, and at the same time there is a lower presence of hydrocolloids and pigments in the extracts.

It is preferred, according to the present invention, to use the residues that occur in hydrocolloid production as a raw material used for human nutrition. This provides optimum utilization of the residue of algae extraction. The order of the extraction processes (extraction with an aqueous and organic solvent) is in principle not significant. However, a prior alginate production is preferred, because following it the relative lipid content in the raw material and thus the yield can be increased, and the presence of hydrocolloids in the lipid extracts is minimized.

As raw material that can be used in the method of the invention, microalgae, which have in the past already been used occasionally for human food in some countries, can also be used according to the invention. These predominantly single cell algae, whose habitat is in fresh water, sea water, or brackish water, are often cultivated in open-field ponds, utilizing sunlight.

In recent years, there have been increasing attempts to ferment single cell algae under defined culture conditions. This algae biomass produced for fermentation is already available on the market, for instance for the species of the Spirulina, Dunaliella and Porphyridium genuses. In addition to these autotrophically cultivated species - that is, species cultivated under sunlight or under artificial light - methods have been developed for producing certain microalgae biomass heterotrophically in closed fermenters at economical cost. All these microalgae from the Cyanophyta, Chrysophyta, Dinophyta, Euglenophyta, Rhodophyta and Chlorophyta phyla, cultivated in open ponds or autotrophically or heterotrophically fermented, can be used according to the invention.

If the residues used according to the invention from hydrocolloid extraction and the microalgae used according to the invention have a moisture content of more than 50 weight % and/or a particle size of more than 50 mm, then this raw material is dried and/or ground prior to the extraction according to the invention, so that the water content of the biomass used in the method of the invention is $\leq$ 50 weight % and the particle size is $\leq$ 50 mm.

A raw material with a water content of from 5 to 50 weight %, in particular 5 to 15 weight %, and a particle size of 0.01 to 50 mm, in particular 0.1 to 1.0 mm, is preferably used.

An organic solvent or a compressed gas is used as the solvent for extraction of the lipids with a high proportion of LCPs. In particular, classic organic solvents and low alcohols with 1 to 6 carbon atoms are used as the organic solvent. Preferably, solvents and alcohols that are miscible with water in any ratio are used. Ethanol can be named as a preferred example. Naturally mixtures of the solvents named may also be used. Preferably, organic solvents that are permitted by the various legal regulations for foods are employed.

As compressed gases, carbon dioxide or propane or a mixture thereof are preferably used. The gas used must be under sufficient pressure and have sufficient temperature to assure that it will be in a liquid or supercritical state. Such compressed gases are characterized by characteristic dissolution properties, particularly for lipophilic ingredients.

To change the extraction properties, other gases or liquids may be admixed with the compressed gas as an entraining agent in a quantity such that the mixture under the extraction conditions is in a uniform liquid or supercritical state. As the entraining agent, a compressed gas that is more-polar or more-nonpolar than the compressed gas used for the extraction, or an organic solvent, may be employed. In this way, the polarity of the extraction agent and thus the dissolution properties thereof can be varied or adjusted.

The extraction with an organic solvent is carried out in particular at a temperature of from 20°C to 65°C, with the upper temperature value naturally depending on the solvent used. Extraction is preferably done at a temperature of approximately 60°C, especially if ethanol is used as the organic solvent.

The extraction is preferably carried out in the form of a batchwise extraction (maceration), percolation, decanter extraction, or countercurrent extraction. The total yields in these types of method may admittedly be less than in an extraction of all the lipids, for instance with the aid of the exhaustive Soxhlet extraction. However, these procedures can be carried out in substantially less time and are thus more economical.

In extraction with a compressed gas, percolation is preferably carried out.

Although in the context of the present application the term organic solvent is used in the method of the invention, it is nevertheless understood to mean both the corresponding water-free solvents and solvents that contain water (up to 30 volume %). Hence, standard solvents can be used without requiring them to be dried beforehand. Nevertheless, a high proportion of water can impair the yield of the desired lipids.

To separate the lipid extract from the extraction liquid (miscella) obtained from extraction with organic solvents, the temperature of the miscella is preferably lowered enough that the lipid extract precipitates out at least in part. If a solvent that is miscible with water in any ratio is used as the solvent, an example being ethanol or isopropanol, then the lipid extract can also be separated by increasing the water content, and this provision can also be

combined with the described lowering of temperature. The organic solvent is then not removed, or is removed only partially, from the miscella. The miscella is mixed with water in a first stage, optionally while being cooled, so that the dissolution capacity of the mixture at the temperature selected is no longer sufficient to keep the lipids in solution. The lipids are then separated from the miscella, for instance with a filter separator.

The liquid solvent can be removed from the resultant solution at standard pressure and high temperatures, and the remaining extract can thus be recovered.

In this separation of the lipid extract from the miscella by increasing the water content and/or by lowering the temperature, the water content of the miscella is preferably increased to 20% to 90%, and in particular to 30 to 50%. The temperature of the miscella is preferably lowered to values of from +20°C to -60°C, and in particular to +5°C to -18°C. It is self-evident that the water content of the solvent originally used, and hence of the miscella, was above the values given prior to the addition of the water. A solvent (such as ethanol) that has a water content of 0 to 20 volume percent, in particular 4 to 15 volume percent, is preferably used.

The situation is similar for the temperature. Hence the temperature of the miscella prior to the lowering of temperature must naturally be above those values to which the temperature is lowered. Preferably the miscella is at a temperature of from 40°C up to the boiling temperature of the solvent used.

Naturally, the total extract can also be recovered from the miscella by evaporating off the solvent. However, by increasing the water content and/or lowering the temperature it is possible for the lipids with the fatty acids of interest here to be precipitated out virtually quantitatively.

From the miscella obtained with the aid of an organic solvent or from the extract obtained from that and freed completely or partially of solvent, extraction can be done once again with a compressed gas, preferably carbon dioxide. In this preferred embodiment, the

extract obtained with the aid of the organic solvent was fractionated into a nonpolar high triglyceride-containing fraction and a polar phospholipid-containing fraction, which optionally after suitable refinement can be used for manifold purposes.

The conditions for the fractionating extraction with the compressed gas are the same as for the extraction with this gas of the algae etc. originally used.

It was surprisingly found that it is possible, by selection of certain raw materials and by certain extraction steps, to obtain a lipid extract or lipid extract fractions with lipids that are rich in certain LCPs. The subject of the invention accordingly also includes a lipid extract or a lipid extract fraction with a content of arachidonic acid of at least 5 weight % and/or with a content of docosahexanoic acid of at least 3 weight %, in terms of the total weight of the fatty acids. It is self-evident that these fatty acids are not available in free form but rather in "bound form" such as triglyceride, glycolipid, phospholipid, etc. By furnishing this lipid extract or these lipid extract fractions, it is possible among other things to furnish a raw material for producing baby foods that contains the arachidonic acid and/or docosahexanoic acid important for the development of the child. Lipids that are rich in these fatty acids are otherwise obtainable only with difficulty or in a commercially unsatisfactory way. The content of arachidonic acid and docosahexanoic acid and in particular the content of these two fatty acids in a lipid extract naturally depends on the choice of raw material used. However, it is sufficient if the lipid extract contains one of these fatty acids in a high proportion, since it can naturally be mixed with other extracts and other ingredients.

The subject of the invention is also a lipid extract or a lipid extract fraction obtainable by the method of the invention.

The LCP-containing lipid extracts or individual lipid fractions (triglycerides, glycolipids, phospholipids, etc., or mixtures of both) according to the invention may, optionally after conventional refinement and stabilization, be used as an additive to the fat content of infant formulas. The term "infant formulas" is understood here to include not

only the usual starting milk formulas for premature and term infants, but also special products that are offered for therapy or prevention of atopic diseases, for instance.

Because of the characteristic proportions of LCP and their emulsifying properties, phospholipid-containing fractions from algae raw materials in particular, after suitable refinement and stabilization, can also be used as an additive in fat emulsions for parenteral nutrition.

The lipid extracts and/or lipid fractions mentioned, and/or the alkyl esters obtained after hydrolysis and re-esterification of the LCPs, may be used in suitable form (such as gelatin capsules) for the prevention of arteriosclerotic diseases and of inflammatory autoimmune diseases.

The LCP-containing fractions and in particular the phospholipid fractions can serve as an active ingredient additive to cosmetic preparations or as starting material to form liposomes, which can also be added to cosmetic preparations.

Particularly the LCP-containing phospholipid fractions, because of their physical-chemical properties, can be used as emulsifiers in the food and cosmetic industries.

Highly purified fractions of the LCP-containing lipids and the free fatty acids and fatty acid esters obtained after hydrolysis and optional re-esterification can be employed as comparison substances (standards) in analysis.

Example 1

Extraction of various raw materials using Soxhlet extraction

A number of different algae species and a number of different residues of alginate and carrageenan production were extracted with the aid of exhaustive Soxhlet extraction, using ethanol (96%; V/V) over a period of 40 hours. The lipids were extracted quantitatively.

The natural mixtures of triglycerides, glyco- and phospholipids and fat-soluble pigments and vitamins have been referred to as complete lipids hereinafter.

The results of these extractions are summarized in Tables 1 and 2. These tables show the respective fatty acid pattern or the various total lipid or fatty acid yields.

The results listed in these Tables 1 and 2 show that the brown and red algae or the residues of alginate and carragheenin production, extracted according to the invention, have a high content of physiologically significant polyunsaturated fatty acids. Arachidonic acid (AA; 20-4 n6) and docosahexanoic acid (DHA; 22-6 n3) can be named primarily as indicative components. These fatty acids may be extracted in quite different proportions from the various raw materials. While arachidonic acid can be extracted from all raw materials in proportions of 5 to 8 weight %, the raw materials of alginate and carragheenin production designated as alginate 3 and 4 are above all characterized by characteristic docosahexanoic acid contents. In the other raw materials, this fatty acid does not occur, or occurs in only very low proportions.

The extraction yields shown in Table 2 demonstrate that the total extractable lipid content of the various algae raw materials ranges between approximately 10 and 70 g/kg of dry composition, and in each case approximately 50% total fatty acids can be recovered. In the residues of alginate production, the proportion of extractable indicative fatty acids (n6-LCP+n3-LCP) ranges up to 7 g/kg of dry substance, and in the native algae up to 6 g/kg of dry substance.

The method according to the invention can be carried out on a commercial scale especially if the two residues alginate 2 and alginate 3 of alginate production, the single-cell oil (microalgae) and the algae flour from Ascophyllum nodosum are used as raw materials in the method of the invention.

The extract obtained by the solvent extraction can be fractionated and purified by conventional processes. Extraction according to the invention with a compressed gas can also ensue.

Table 1

Fatty acid pattern of microalgae, macroalgae, and residues of alginate production by means of ethanol-Soxhlet extraction in weight % of the total fatty acid pattern

Name	Micro	Alginate 1	Alginate 2	Alginate 3	Alginate 4	Fucus	Asco.	Lam.	Macro.
12-0	0.50	0.78	0.84	0.20	1.06	0.08	0.15	0.51	0.32
14-0	0.49	9.37	6.94	10.04	6.16	11.76	10.40	7.06	13.90
t14-1 n5	0.06	0.27	0.25	0.27	0.15	0.08	0.08	0.24	0.15
14-1 n5	0.00	0.11	0.09	0.23	1.66	0.09	0.12	0.05	0.82
15-0	0.42	0.51	0.30	0.41	0.55	0.39	0.33	0.43	0.74
16-0	49.66	23.53	17.39	13.40	27.92	19.27	12.40	22.39	29.90
t16-1 n7	3.97	0.25	0.21	0.19	1.33	0.12	0.12	0.00	2.55
16-1 n7	0.45	3.08	3.00	1.45	3.46	1.49	1.28	4.05	3.16
17-0	0.39	0.16	0.11	0.18	0.25	0.15	0.17	0.14	0.23
18-0	2.97	0.97	0.63	0.66	1.80	0.82	0.93	1.11	1.51
t18-1 n9	0.06	0.03	0.03	0.00	0.23	0.00	0.02	0.02	0.14
18-1 n9	1.30	29.34	22.64	44.34	22.92	36.33	43.80	20.01	22.40
18-2 n6	6.94	7.37	6.15	7.44	3.32	8.74	8.00	5.83	3.87
18-3 n3	0.78	3.40	5.69	1.74	0.78	2.64	2.30	6.00	2.00
18-4 n3	0.00	3.31	11.27	1.00	1.40	2.15	1.43	9.92	2.03
20-0	0.00	0.75	0.43	0.32	2.00	0.43	0.29	0.49	1.13
20-1 n9	0.00	0.04	0.05	0.09	0.80	0.05	0.11	0.02	0.04
20-2 n6	1.09	0.29	0.51	1.25	0.15	0.22	1.37	0.14	0.04
20-3 n6	0.98	0.45	0.41	0.58	0.48	0.76	0.59	0.30	0.33

20-4 n6	12.06	7.20	8.41	5.22	5.01	7.34	8.05	7.72	7.85
20-3 n3	0.00	0.08	0.16	0.03	0.02	0.06	0.27	0.14	0.03
20-5 n3	8.19	5.54	10.62	1.87	1.18	3.15	3.06	9.82	2.41
22-0	0.00	0.08	0.04	0.15	0.03	0.15	0.12	0.02	0.09
22-1 n9	0.00	0.01	0.01	0.14	0.68	0.02	0.30	0.37	0.04
24-0	0.00	0.09	0.11	0.21	0.00	0.14	0.20	0.05	0.20
24-1 n9	0.00	0.55	0.35	1.70	0.01	0.89	1.19	0.02	0.11
22-6 n3	0.00	0.03	0.00	3.56	7.40	0.17	0.08	0.10	0.05
n.i.		9.69	3.38	3.34	9.24	2.50	2.74	3.05	3.85
trans FS	4.63	0.55	0.49	0.46	1.72	0.2	0.22	0.26	2.84
TOTAL	100	100	100	100	100	100	100	100	100

Micro = microalgae, such as Porphyridium cruentum; Alginates 1-4 = various residues from alginate production; Fucus = Fucus serratus; Asco. = Ascophyllum nodosum; Lam. = Laminaria digitata; Macro. = Macrocystis pyrifera; n. i. = not identified; trans FS = trans-fatty acids.

Table 2

Total extract, lipid extract and fatty acid yields of microalgae, macroalgae and residues of alginate production from ethanol-Soxhlet extraction in g/kg of raw material dry substance

	Micro	Alginate 1	Alginate 2	Alginate 3	Alginate 4	Lam.	Macro.	Fucus	Asco.
GE	233.7	77.22	98.25	111.32	52.28	130.64	61.98	246.92	157.03
LE	20.7	55.60	73.51	60.36	22.16	22.45	11.25	46.15	73.74
Ges.-FS	10.1	28.07	34.81	34.00	7.54	9.78	5.11	27.26	42.19
%FS in LE	48.8	50.49	47.36	56.33	37.48	43.59	45.40	59.07	57.20
n6-LCP	14.14	2.28	3.36	2.48	0.47	0.82	0.44	2.93	4.54
n3-LCP	8.19	1.62	3.88	1.92	0.71	1.02	0.13	0.95	1.48
20-4 n6	2.49	2.07	3.03	1.84	0.42	0.78	0.42	2.05	3.49
20-5 n3	1.69	1.59	3.83	0.66	0.10	0.99	0.13	0.88	1.33
22-6 n3	0.00	0.01	0.00	1.25	0.61	0.01	0.00	0.05	0.03

Micro = microalgae, such as Porphyridium cruentum; Alginates 1-4 = various residues from alginate production; Lam. = Laminaria digitata; Macro. = Macrocystis pyrifera; Fucus = Fucus serratus; Asco. = Ascophyllum nodosum; GE = total extract; LE = lipid extract after removal of the hydrophilic ingredients ("FOLCH"); Ges.-FS = total fatty acids; n6-LCP = total of n6 fatty acids with 20 and more carbon atoms in the acyl radical; n3-LCP = total of n3 fatty acids with 20 and more carbon atoms in the acyl radical; 20-4 n6 = arachidonic acid; 20-5 n3 = eicosapentaenoic acid; 22-6 n3 = docosahexaenoic acid.

The extract is preferably freed of solvent completely by distillation at reduced pressure, and then subjected to the usual purification processes in the production of food fats in industry, such as bleaching, degumming and deodorization. The bleaching agent can already be added before complete removal of the solvent has occurred.

Example 2

Extraction of lipids from the alga Ascophyllum nodosum with 90% ethanol

Flour of the alga Ascophyllum nodosum was used as the raw material.

Characterization of the Ascophyllum nodosum raw material:

Water content: 9.6%

Particle size Screen mesh width (mm)	% distribution
0.5	0.2
0.25	0.4
0.1	75.4
0.05	23.1
>0.05	0.9

The solvent extraction over four hours was performed with ethanol (90%; V/V) at various temperatures. In the apparatus used, the process was a single-stage percolation done on a laboratory scale. The ratio between the extraction agent used and the raw material dry substance was approximately 4:1 (weight/weight).

In comparison with the Soxhlet extraction (see Table 3), in which an extraction time of 40 hours was adhered to, the yield of the four-hour percolation was uniformly quite high. It demonstrated that increasing the extraction temperature leads to an improved yield in all the extract ingredients. Further optimization of the yield in the extraction of this raw material is attainable by further increasing the ratio between the solvent and the raw material or by changing the course of the process in the direction of countercurrent extraction (see Example 3).

Table 3

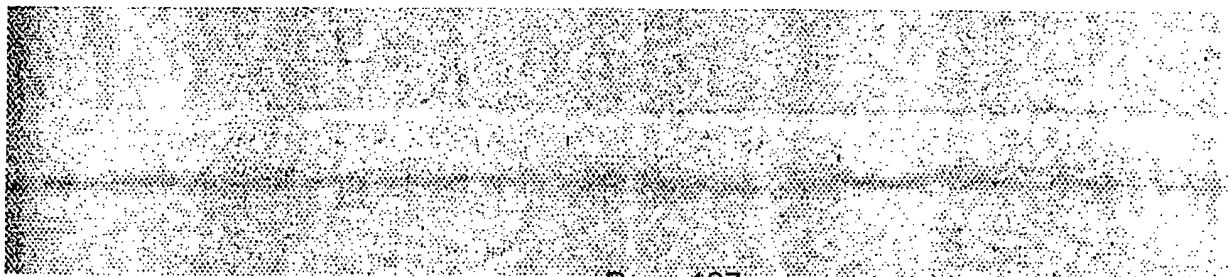
Extraction yields in percolation, in percent of raw material dry substance

Temp. (°C)		20	30	40	50	60	Soxhlet
GE	%	8.06	11.01	14.34	16.54	17.89	15.7
FS	%	0.93	1.18	1.25	1.34	1.41	4.22
20-4 n6	%	0.09	0.11	0.12	0.13	0.14	0.35
20-6 n3	%	0.05	0.06	0.06	0.07	0.08	0.13
Concen-tration	%	2.5	2.3	3.1	3.4	3.7	

GE = total extract; FS = fatty acid yield; 20-4 n6 = yield of arachidonic acid; 20-5 n3 = yield of eicosapentanoic acid

The results of the one-stage percolation test shown in Fig. 3 show that with this experimental apparatus, yields of 41% of the arachidonic acid fractions and 58.5% of the eicosapentanoic acid fractions are attained, compared with the Soxhlet extraction. By the percolation process, it was possible to recover a total of 33.4% of the maximum extractable fatty acids. The value for the total extract yield, which is elevated compared with Soxhlet extraction, can be ascribed to the higher water content (10%) of the ethanol used in the percolation and the resultant more-intensive extraction of hydrophilic substances.

From Table 3, it is also clear that for the extraction of the flour of the alga Ascophyllum nodosum with 90% ethanol, an extraction temperature of approximately 60°C is to be sought, if the method is to be optimized in terms of the miscella concentration, that is, the solvent consumption. Naturally comparable yields are also attainable at lower temperatures and with a correspondingly higher amount of solvent.



Example 3

Staged countercurrent extraction of a residue from alginate production with 90% ethanol

In this example, a raw material was used that was already used to recover alginate (alginate 1). This residue is a mixture of various species of brown algae. The algae residues were dried and ground after the alginate production process, so that in terms of particle size and water content they are approximately equivalent to the flour of the alga Ascophyllum nodosum (see Example 2).

The extraction method of staged countercurrent extraction employed is equivalent to the process carried out on an industrial scale. In contrast to percolation (Example 1), the extract concentration of the miscella in multistage countercurrent extraction fluctuates around a mean value. The more stages that are used and the shorter the dwell time of the raw material in the apparatus, the less pronounced this fluctuation is. With this method, by comparison with percolation, identical yields can be attained, using only a fraction of the solvent, or higher yields can be attained using similar quantities of solvent.

In the example chosen, a four-stage experimental apparatus was chosen. The extraction temperature was 20°C; the ratio between the quantity of extraction agent used and the raw material dry substance was 2:1.

Despite the comparatively simple apparatus (30 stages and more are not unusual in industrial systems), it was possible in this example to attain yields of 66% of arachidonic acid, 72.5% of eicosapentanoic acid and 45.7% of the total fatty acids, by comparison with the Soxhlet extraction.

Table 4

Yields of the multistage countercurrent extraction of alginate 1 compared with Soxhlet extraction, in weight % in terms of the raw material dry substance

Yield	Countercurrent extraction	Soxhlet extraction
Total extract	5.92	7.72
Total lipid extract	4.24	5.56
Total fatty acids	1.28	2.80
Arachidonic acid (20-4 n6)	0.13	0.20
Eicosapentanoic acid (20-5 n3)	0.12	0.16

If these values are compared with the results of the percolation in Example 2, the advantages of countercurrent extraction become clear. Although in countercurrent extraction only 50% of the quantity of solvent was used and the extraction temperature was only 20°C, the yields in terms of total fatty acid and arachidonic acid are nevertheless clearly superior to that of percolation.

Example 4

Extraction of flour of the alga Ascophyllum nodosum with compressed carbon dioxide

For extraction with compressed gases, the dried, ground raw material of native algae or of residues from alginate and carragheenin production is used. The water content of the starting material is typically between 5 and 50 weight % and preferably between 10 and 20 weight %. The particle size of the material is 0.01 mm to 50 mm, preferably 0.1 mm to 0.3 mm.

The raw material used is the algae flour of Ascophyllum nodosum already employed in Example 2. The extraction was carried out with compressed carbon dioxide (150 bar,

35°C, approximately 11 kg of CO_2/kg of raw material). Under these conditions, at least 90% of the total lipids extractable with this solvent can be recovered.

Table 5

Extract and fatty acid yields of high-pressure extraction of Ascophyllum nodosum in comparison with ethanol Soxhlet extraction, in weight % of the raw material dry substance

Yield	High pressure extraction	Soxhlet extraction
Total lipid extract	2.50	7.30
Total fatty acids	2.10	4.20
Arachidonic acid (20-4 n6)	0.18	0.35
Eicosapentaenoic acid (20-5 n3)	0.08	0.13

In Table 5, the maximum attainable yields in terms of the raw material mentioned with each method are compared. Soxhlet extraction here stands for processes of solvent extraction using ethanol overall because in principle this represents nothing more than a repeated process of percolation/maceration.

To enable evaluating the quality of the extracts obtained, the fatty acid pattern of the extracts recovered by the two methods are compared in Table 6.

Table 6

Comparison of the fatty acid spectra of a high-pressure extract and a Soxhlet extract of Ascophyllum nodosum; figures given as weight % of the total fatty acids

Fatty Acids	High-pressure Extract	Soxhlet Extract
14-0	5.58	10.4
14-1 n5	0.15	0.12
16-0	7.46	12.4
16-1 n7	0.94	1.28
18-0	0.97	0.93
18-1 n9	53.8	43.8
18-2 n6	9.56	8.0
20-0	0.34	0.29
18-3 n3	1.58	2.3
20-1 n9	0.48	0.11
20-2 n6	1.77	1.37
22-0	2.88	0.12
20-3 n6	0.91	0.59
22-1 n9	0.44	0.30
20-4 n6	7.26	8.05
20-5 n3	2.18	3.06

The results in Table 6 clearly show that the solvent extract of Ascophyllum nodosum has only a slightly more favorable composition in terms of the proportion of arachidonic acid and eicosapentanoic acid, compared with the high-pressure extract. Since with the aid of high-pressure extraction, it is primarily nonpolar lipids (triglycerides) that are extracted, it must be presumed that the indicative fatty acids also occur in high proportions in these lipids.

Example 5

Separation of the lipid fraction from the miscella by increasing the water content

Separation of the lipid fraction from the miscella by increasing the water content was carried out in the example of the miscella obtained by staged countercurrent extraction of alginate 2 with 90% ethanol. Five aliquot portions were taken from the miscella and adjusted to various water contents, beginning with 10% water in the starting miscella (see Table 7). The extracts precipitated after the addition of water were recovered by centrifuging, decanting of the remainder, and ensuing drying.

Table 7 below clearly shows the relationship that exists between the water content of the miscella and the yields of the precipitated lipid fractions. The table shows the lipid yields obtained with a comparison extract that was recovered from by complete evaporation off of the solvent of an aliquot quantity of the starting miscella. The yields are shown relatively in percent of comparison extract (comparison extract = 100%).

Table 7

Lipid yields from the miscella after precipitation by increasing the water content, in percent of the comparison extract (equals 100%); values for water content in absolute percent

Water Content	25.0	35.7	43.75	50	55
GE	46.06	78.63	84.76	87.31	88.73
LE	48.28	74.92	80.68	84.8	88.32
FS	51.59	90.07	93.24	94.61	84.7
20-4 n6	64.56	100.2	102.9	103.1	97.58
20-5 n3	57.49	97.94	102.32	104.41	95.62

GE = total extract; LE = lipid extract; FS = fatty acids; 20-4 n6 = arachidonic acid; 20-5 n3 = eicosapentanoic acid

The values in Table 7 show that increasing the water content of the miscella from 10% to 35% is already sufficient for the lipids, in which the indicative fatty acids arachidonic acid and eicosapentanoic acid are localized, to be virtually quantitatively precipitated out. The values over 100% are suspected to be the result of the fact that because of the greater purity of the lipid fractions after precipitation in comparison with the comparison extract, the fatty acids can be more completely converted into the derivatives that are determinable by gas chromatography.

The method of lipid precipitation from the miscella employed here, by increasing the water content, also has the following advantages in terms of the process:

1. As a result of the major increase in the water content of the miscella, prepurification of the extract takes place, because hydrophilic substances, which at a 10% water content of the extraction agent are already co-extracted, remain in solution and in this way need not be removed from the total lipid extract only afterward by complicated processes.

2. Alginates or carragheenins, which are extracted primarily from native algae in sometimes quite major quantities with 90% ethanol, can cause problems in solvent recovery in modern evaporator systems (such as downflow evaporators). However, if the lipid extract is obtained by adding water from the miscella, then the excess water keeps the hydrophilic hydrocolloids in solution, so that the separation of the solvent from the remaining miscella can be carried out without problems.

In order to check whether a selective precipitation of the lipids from the miscella and hence a variation in the fatty acid pattern of the lipids occurs from the water addition, the extract precipitated out with a miscella water content of 55% and the comparison extract were studied by gas chromatography as an example.

Extract 1 listed in Table 8 below was obtained from the starting miscella by increasing the water content to 55%, while the comparison extract was obtained by evaporating the solvent out of the starting miscella.

Table 8

Fatty acid pattern of an extract precipitated out with a 55% water proportion, compared with the total extract; figures given in weight % of the total fatty acids

Fatty Acids	Extract 1	Comparison Extract
14-0	8.26	7.9
14-1 n5	0.76	0.69
16-0	19.73	18.9
16-1 n7	4.28	4.32
18-0	0.43	0.39
18-1 n9	24.56	22.46
18-2 n6	7.88	7.43
20-0	0.37	0.34
18-3 n3	6.97	6.77
20-1 n9	0.17	0.15
20-2 n6	0.27	0.26
20-3 n6	0.4	0.37
22-1 n9	0.08	0.07
20-4 n6	11.44	10.72
20-5 n3	10.56	10.01
22-6 n3	0.46	0.36

The results of the fatty acid analysis in Table 8 show that the fatty acid spectra of the two extracts are not substantially different. The proportions of the indicative fatty acids arachidonic acid and eicosapentaenoic acid, at 10 to 11 weight %, also virtually agree. This

result confirms the findings already shown in Table 7, that by purposeful addition of water a quantitative precipitation of the lipids and fatty acids from the raw miscella is possible.

Example 6

The separation of the neutral lipid extract by extraction with compressed gases is described below.

In a suitable system, the miscella obtained with organic solvents (preferably ethanol) or an extract freed completely or partially of the solvent is brought into contact in an extraction autoclave with compressed gases, preferably carbon dioxide. An entraining agent can be added in metered fashion to the compressed gas in order to purposefully adjust the extraction properties.

If the liquid solvent was removed completely from the extract and no entraining agent was admixed with the extraction gas, then the triglycerides, carotenoids, chlorophylls and phytosterols are dissolved selectively out of the extract. By a suitable selection and metered addition of the entraining agent or incomplete separation of the liquid solvent from the extract, the dissolution properties of the extraction gas can be expanded substantially.

By reducing the pressure and/or raising the temperature of the extraction gas, its solubilizing capacity is reduced in stages. In each of these stages, a certain portion of the dissolved lipids occurs as an extract fraction. In this way, fractionation of the lipids into one nonpolar and one polar fraction primarily takes place. The nonpolar lipid fraction can then, in the course of a multistage precipitation, be subjected to further fractionation.

This separation will be described in detail in terms of a CO_2 high-pressure countercurrent extraction of a miscella.

An aliquot of a miscella that was obtained by maceration of alginate 2 with 90% ethanol is extracted in countercurrent with compressed carbon dioxide (150 bar, 35°C) in a

stripper column. After the extraction, the compressed gas is expanded in a precipitation autoclave to 20 bar, so that the lipid dissolved in the fluid occurs in the form of extract. The fraction not soluble in the fluid collects at the bottom of the column. It comprises polar lipids and the non-lipophilic remaining extract. The fill level of the separator and of the stripper column can be monitored visually during the extraction via viewing windows. In the course of the extraction, when a certain fill level is attained, both fractions can be removed via a valve.

The maceration was carried out in this example as a one-stage batch extraction at 35°C for 20 hours. The miscella concentration prior to high-pressure extraction was 1.37% in terms of the total extract and 1% in terms of the lipid extract. Some of the miscella obtained by the maceration was removed and used for the high-pressure extraction. After the high-pressure extraction, 41.3% of the total extract and 50.9% of the lipid extract could be found in the separator.

Gas chromatography investigation of the extracts in the separator and prior to the high-pressure extraction

In order to be able to evaluate whether the neutral lipid extract obtained by high-pressure extraction differs in the composition of the fatty acids compared with the total extract of the fractionation, a gas chromatography analysis of the fatty acid pattern of the extract was carried out before and after the high-pressure extraction.

Table 9

Fatty acid pattern of a lipid extract of alginate 2, obtained by maceration, before and after high-pressure extraction (HD-E) in weight % of the total fatty acids

Fatty Acids	Before HD-E	After HDE i.S.
14-0	8.25	7.65
t14-1 n5	0.07	0.00
14-1 n5	0.41	0.27
15-0	0.36	0.11
16-0	20.17	19.42
t16-1 n7	0.27	0.23
16-1 n7	3.56	3.42
17-0	0.15	0.11
18-0	1.04	0.61
t18-1 n9	0.07	0.02
18-1 n9	26.02	22.85
18-2 n6	7.10	6.18
19-0	0.00	0.00
18-3 n3	4.68	6.18
18-4 n3	9.64	12.47
20-0	0.61	0.39
20-1 n9	0.14	0.05
20-2 n6	0.49	0.42
20-3 n6	0.50	0.35
20-4 n6	7.84	7.60
20-3 n3	0.14	0.15
20-5 n3	7.68	10.37

22-0	0.05	0.07
22-1 n9	0.11	0.17
24-0	0.07	0.06
24-1 n9	0.10	0.28
22-06 n3	0.11	0.03
Total	100	100

HD-E = high-pressure extraction; FS = fatty acids; i.S. = in the separator

Comparison of the fatty acid spectra of the lipid extracts before and after the high-pressure extraction shows that the fatty acid pattern of the neutral lipid fraction does not differ substantially from the total extract. The proportion of the physiologically significant fatty acids arachidonic acid (20-4 n6) and eicosapentaenoic acid (20-5 n3) in the neutral lipid extract does not vary toward the negative as a result of the high-pressure extraction, but in the case of the eicosapentaenoic acid on the contrary even rises.

The results of this experiment show that the LCPs are localized in substantial proportions in the neutral lipids of the residues from alginate production.

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(19) (CA) **CANADIAN PATENT** (12)

(54) METHOD FOR THE PROCESSING OF KRILL TO PRODUCE PROTEIN, LIPIDS AND CHITIN

(72) Rogozhin, Sergei V.;
Vainerman, Efim S.;
Burmistrova, Ljubov M.;
Davidovich, Jury A.;
Ryashentsev, Vladimir J.;
Kulakova, Valentina K.;
Lagunov, Lev L.;
Bykov, Vladimir P.,
USSR

(73) Granted to Institut Elementoorganicheskikh Soedineny Akademii Nauk SSSR, USSR Vsesojuzny Nauchno-Issledovatelsky Institut Rybnogo Khozyaistva I Okeanografii, VNIRO, USSR

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1098900 ABSTRACT OF THE DISCLOSURE APR 7 1981

A1

The method for the processing of ~~kriol~~ krill to produce protein, lipids and chitin comprises emulsification of lipids by intensively stirring ~~kriol~~ krill in an aqueous medium. The resultant emulsion of lipids is separated from the ~~kriol~~ krill mass and from the ~~kriol~~ krill mass proteins are extracted at a pH of 10 to 12. The alkaline extract of proteins is separated from chitin integuments and protein is separated therefrom.

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

1. A method for the processing of krill to produce protein, lipids and chitin which comprises emulsification of lipids of krill in an aqueous medium; separation of the resultant emulsion of lipids from the krill mass; alkaline extraction of proteins from the krill mass at a pH of 10 to 12; separation of the alkaline extract of proteins from chitin integuments; separation of protein from the alkaline extract.

2. A method as claimed in claim 1, wherein emulsification is carried out in the presence of mineral salts.

3. A method as claimed in claim 1, wherein emulsification is carried out at a pH of the medium of 4.5 to 5.0.

METHOD FOR THE PROCESSING OF KRILL TO PRODUCE PROTEIN, LIPIDS AND CHITIN

The present invention relates to methods for the processing of krill to produce protein, lipids and chitin. Krill is a prospective source of food protein and other practically useful products such as chitin and lipids which find wide application in different branches of the national economy -- the food industry, textile and paint and varnish industry, in agriculture and medicine.

Known in the art is a method for the production of a proteinaceous nutritive substance from krill residing in comminuting and pressing fresh or frozen and then defrosted krill. The liquid separated during pressing is heated for 10 to 15 minutes at a temperature of 90 to 95°C for coagulation of proteins contained therein. The proteinaceous coagulate is separated from the broth by filtration or centrifugation to produce a mass which is used in the USSR under a trade name of the Okean protein paste.

A disadvantage of said method for the processing of krill is loss of nutritive substances, particularly protein, and an insufficiently full utilization of other components of krill. The broth containing a considerable amount of nutritive substances is not processed and is poured off. The yield of protein is 35 to 40%. It should be pointed out that the Okean paste is a perishable product and should be stored only when frozen at a temperature not exceeding -18°C for not more than 12 to 14 months. The thermally denatured protein contained in the Okean paste possesses low functional properties (foam-forming and gel-forming properties, a water-holding capacity, etc.) which makes its processing and use difficult. The cake formed after pressing comprising a portion of the starting

proteins, lipids and chitin integuments can at present be processed and used only as feed meal.

Known in the art is a method for the production of a protein concentrate from frozen krill kept at a temperature of -20°C comprising defrostation, comminution of krill, extraction with isopropanol with subsequent removal of the solvent, and drying under vacuum at 70°C.

Using the present method a proteinaceous concentrate is produced with a content of protein of 710 to 775%, lipids of 0.3%, and chitin of 5.8 to 6.4% (as calculated for dry substance). Said method has the following disadvantages. The use of organic solvent makes the production more difficult. In addition, the solvent itself and the process for the removal thereof may deteriorate the quality of the protein. The proteinaceous concentrate has a comparatively low content of protein and a high chitin content.

It is an object of the present invention to develop such a method for the processing of krill which would make it possible to produce protein, lipids and chitin with a high yield and quality.

The method for the processing krill to produce protein, lipids and chitin, according to the invention, is characterized in comprising emulsification of lipids of krill in an aqueous medium; separation of the emulsion of lipids from the krill mass; alkaline extraction of proteins from the krill mass at a pH of 10 to 12; separation of the protein extract produced from chitin integuments; separation of protein from the protein extract.

The invention makes it possible to obtain a protein product with a content of protein of up to 95% by weight as calculated for dry substance.

According to the invention, the first stage of the

processing of krill provides for extraction of lipids. This extraction of lipids is effected by emulsification using various techniques such as intensive stirring in an apparatus with a stirrer, or an ultrasonic method. Used as a medium in which emulsification is conducted is water or aqueous solutions of salts. To reduce losses of protein in the process of emulsification the pH of the emulsifying medium should be maintained within 4.5 to 5.0. In emulsification lipids are separated with a yield of up to 95% by weight.

The krill after separation of lipids therefrom is treated with an alkaline solution with a pH of 10 to 12 for extraction of proteins therefrom. A two-phase system is formed comprising an aqueous-alkaline solution containing protein, and a solid residue containing chitin integuments and other insoluble substances. The aqueous-alkaline solution containing protein is separated from the solid residue by filtration or centrifugation. Protein is separated from the resultant aqueous-alkaline solution by various mehtods, for example, by precipitation with alcohol or ultrafiltration, precipitation in the isoelectric point, or thermal coagulation. The isoelectric precipitation is carried out by food acids at a pH of 4 to 5. A curdled, easily settling precipitate of protein is formed which is separated and washed with 2 to 5 volumes of water. The washed precipiate is dried. As a result a product is obtained with a protein content of up to 95% by weight as calculated for dry substance.

Thus, the proposed method for the processing of krill makes it possible to produce such valuable substances as protein, lipids and chitin.

The simple technology and the availability of the reactants used make the process commercially profitable.

For a better understanding of the present invention

examples are presented below.

Example 1

In an apparatus with a capacity of 10 l provided with a stirrer there is placed 1 kg of krill which is filled with water and stirred at 1,000 rpm for 0.3 hour. The resultant emulsion of lipids is separated from the krill mass by filtration through a stainless steel screen having a mesh size of 1x1 mm. The krill mass is transferred to the vessel with a stirrer into which there is added 3 l of an aqueous solution of NaOH of such a concentration as to reach a pH of the mixture of 10 and stirred for half an hour. When krill is treated with alkali extraction of proteins takes place. The resultant extract of proteins is separated from the insoluble residue of chitin integuments by filtration through a metal screen with a mesh size of 1x1 mm and centrifuged at 25,000 rpm for 0.15 hour to remove impurities. To the purified extract of proteins there is added while stirring a 1-mole solution of HCl to reach a pH of 4.5, protein being precipitated. The precipitate is left to settle for 3 hours, thereafter it is separated from the liquid, washed with 3 liters of water and dried lyophilically. The protein product obtained in an amount of 50 g is a pale-pink odorless powder, having a moisture content of 10% by weight and comprising 85% by weight of protein and 2% by weight of lipids.

The residue of krill produced after separation of the extract of proteins is pressed to remove moisture and dried to produce 17 g of chitin integuments.

Example 2

The processing of krill is carried out in the same manner as in Example 1, except that emulsification of lipids is conducted in a 0.15 mole aqueous solution of sodium chloride at a pH of 4.5. The protein product obtained in an amount of 54 g has a moisture content of 12% and comprises 80% by weight of protein and 3% by weight of lipids, and 20 g of

chitin integuments.

Examples 3

The processing of krill is conducted in the same manner as in Example 1, except that emulsification of lipids is carried out for half an hour, and protein is precipitated from the alkaline extract by adding thereto a 1 mole solution of acetic acid. The resultant protein precipitate is washed with 5 volumes of water to produce 60 g of a protein product having a moisture content of 8% and comprising 85% by weight of protein, 5% by weight of lipids and 18 g of chitin integuments.

Example 4

The processing of krill is carried out in the same manner as in Example 1, except that precipitation of protein from the alkaline extract is conducted by adding thereto a 0.8 mole solution of sulfuric acid. The resultant precipitate of protein is washed with 3 volumes of water to produce 54 g of a protein product having a moisture content of 11% and comprising 80% by weight of protein and 5% by weight of lipids, and 24 g of chitin integuments.

Example 5

The processing of krill is conducted in the same manner as in Example 1, except that emulsification of lipids is conducted in an aqueous solution of salts -- 0.2 mole of NaCl; 0.03 mole of $MgCl_2$; 0.01 mole of $MgSO_4$; and 0.005 mole of $CaSO_4$.

The protein product obtained in an amount of 60 g has a moisture content of 10% and comprises 82% by weight of protein, 4.2% by weight of lipids, and 20 g of chitin integuments.

liquid, washed with 3 liters of water and dried lyophilically. The protein product obtained in an amount of 50 g is a pale-pink odorless powder, having a moisture content of 10% by weight and comprising 85% by weight of protein and 2% by weight of lipids.

A

The residue of ~~kriol~~ krill produced after separation of the extract of proteins is pressed to remove moisture and dried to produce 17 g of chitin integuments.

Example 2

The processing of ~~kriol~~ krill is carried out in the same manner as in Example 1, except that emulsification of lipids is conducted in a 0.15 mole aqueous solution of sodium chloride at a pH of 4.5. The protein product obtained in an amount of 54 g has a moisture content of 12% and comprises 80% by weight of protein and 3% by weight of lipids, and 20 g of chitin integuments.

Example 3

The processing of ~~kriol~~ krill is conducted in the same manner as in Example 1, except that emulsification of lipids is carried out for half an hour, and protein is precipitated from the alkaline extract by adding thereto a 1 mole solution of acetic acid. The resultant protein precipitate is washed with 5 volumes of water to produce 60 g of a protein product having a moisture content of 8% and comprising 85% by weight of protein, 5% by weight of lipids and 18 g

of chitin integuments.

Example 4

A The processing of ~~kriol~~ krill is carried out in the same manner as in Example 1, except that precipitation of protein from the alkaline extract is conducted by adding thereto a 0.8 mole solution of sulfuric acid. The resultant precipitate of protein is washed with 3 volumes of water to produce 54 g of a protein product having a moisture content of 11% and comprising 80% by weight of protein and 5% by weight of lipids, and 24 g of chitin integuments.

Example 5

The processing of ~~kriol~~ krill is conducted in the same manner as in Example 1, except that emulsification of lipids is conducted in an aqueous solution of salts -- 0.2 mole of NaCl; 0.03 mole of $MgCl_2$; 0.01 mole of $MgSO_4$; and 0.005 mole of $CaSO_4$.

The protein product obtained in an amount of 60 g has a moisture content of 10% and comprises 82% by weight of protein, 4.2% by weight of lipids, and 20 g of chitin integuments.

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Patent Abstracts of Japan

PUBLICATION NUMBER : 08198754
PUBLICATION DATE : 06-08-96

APPLICATION DATE : 23-01-95
APPLICATION NUMBER : 07025822

APPLICANT : YAKULT HONSHA CO LTD;

INVENTOR : OWAYA HIDEYUKI;

INT.CL. : A61K 31/66

TITLE : AGENT FOR IMPROVING CEREBRAL FUNCTION

ABSTRACT : PURPOSE: To obtain a cerebral function improving agent containing phosphatidyl-L-serine originated from the brain of an animal except a cattle, capable of being massively produced at a low cost, without a problem on the aspects of the cost and supply, and capable of improving the cerebral functions.

CONSTITUTION: This improving agent contains phosphatidyl-L-serine originated from the brain of an animal (e.g. pig, sheep, chicken) except cattle or its salt as an active ingredient. The salt of the phosphatidyl-L-serine is preferably Na salt or K salt. The phosphatidyl-L-serine is obtained e.g. by extracting a swine brain or an ovine brain with acetone, filtering the filtrate, extracting the residue with petroleum ether, vacuum-drying the extract, dissolving the dried product in ethyl ether, adding ethyl alcohol to the solution to produce precipitates, dissolving the precipitates in chloroform, and subsequently purifying the solution with silica gel column chromatography.

Patent Abstracts of Japan

PUBLICATION NUMBER : 04057853
PUBLICATION DATE : 25-02-92

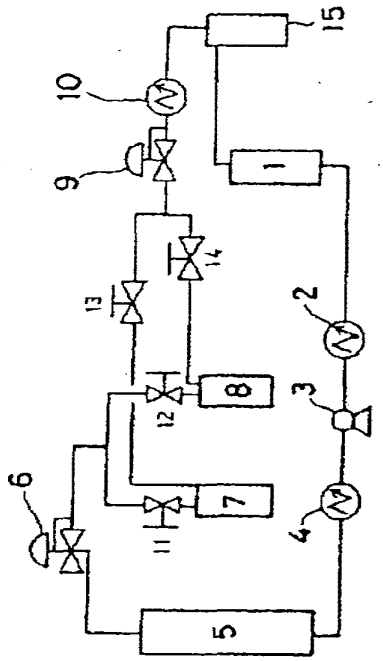
APPLICATION DATE : 28-06-90
APPLICATION NUMBER : 02170549

APPLICANT : ITANO REITOU KK;

INVENTOR : TANI TOSHIFUMI;

INT.CL. : C09B 61/00

TITLE : METHOD FOR EXTRACTING AND SEPARATING COLORING MATTER FROM KRILL



ABSTRACT : PURPOSE: To prepare a reddish orange coloring matter having a high safety in a high concn. by extracting, with CO_2 in a supercritical state, krill shells of which the protein has been decomposed by a protease.

CONSTITUTION: Krill shells are treated with a protease to decompose the protein in the shells and the treatment product is filtered. The residue of filtration is dried to give treated shells having a water content of 6-8% and a mean particle size of 200 μm or lower. The treated shells are put into an extraction vessel 5. An extractant comprising a liq. CO_2 in an amt. of 30-40 pts.wt. based on one pt.wt. treated shells having a coloring matter concn. of 30 mg/100 g is supplied through a supercooling apparatus 2 to a pump 3, pressurized at the pump 3 to 100-250 kg/cm^2, heated with a heat exchanger 4 to 35-40°C to bring it into a supercritical state, and transferred to the extraction vessel 5 to extract an oil in the treated shells. After the pressure of the oil-contg. CO_2 in the supercritical state is reduced to 40-60 kg/cm^2 with a pressure reducing valve 6, the CO_2 is delivered through a selector valve 11 to the first separating vessel 7 to separate the oil, and recycled through a selector valve 13, a pressure reducing valve 9, a condense 10, a water separator 15, and a storage vessel 1 to the extraction vessel 5. Then, selector valves 11 and 13 are closed while selector valves 12 and 14 are opened, and the CO_2 contg. the coloring matter is transferred to the second separating vessel 8, where the CO_2 is evaporated to give a coloring matter with a concn. of 2000-10000 mg/100g.

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(71)(72) Applicants and Inventors: HELLGREN, Lars, Gustav, Inge [SE/SE]; Bronsgjutargatan 13, S-421 63 Västra Frölunda (SE). MOHR, Viggo [NO/NO]; St. Jo/rgensveita 6a, N-7000 Trondheim (NO). VINCENT, Jan, Gustav [SE/SE]; Linnégatan 31, S-114 47 Stockholm (SE).

(74) Agents: KUMMELSTEN, Per, Arne et al.; Uppsala Patentbyrå, Box 9039, S-750 09 Uppsala (SE).

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(54) Title: ENZYME COMPOSITION FOR THERAPEUTICAL AND/OR NON-THERAPEUTICAL CLEANING, THE USE THEREOF AND PREPARATION OF THE COMPOSITION

(57) Abstract

An enzyme composition, containing an effective amount of an enzyme preparation which degrades contaminants of biological origin, for use as a therapeutical and/or non-therapeutical agent, a method for its preparation and a method for the therapeutical and non-therapeutical cleaning of living and dead material. The enzyme preparation used is derived from an aquatic animal of the order *Euphausiaceae* or from a fish. Among fishes those of the genus *Mallotus* are preferred.

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Enzyme composition for cleaning, the use thereof and preparation of the composition

Field of the invention

This invention relates to a composition which contains a digestive and/or tissue enzyme preparation from an aquatic animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the fishes. The composition is used in order to clean living or dead material by degrading and removing therefrom contaminants of biological origin or degradation products of such contaminants. The invention also provides a method for removing said contaminants from said material and a method for the preparation of a composition to be used according to the invention.

As used in the specification and in the claims, the term enzyme means an active enzyme if not otherwise specified.

The invention relates to both the therapeutical cleaning of mammals and to the non-therapeutical cleaning in general. The concept cleaning is hence used in its broadest meaning, i.e. cleaning of dead as well as of living material such as different types of textiles, hair, furs, skins, plastics, leather, nails, ears, mirrors, glass, porcelain, denture prosthesis, metals, stones, teeth, facades, downs, works of art such as paintings, etc. Cleaning also includes cleaning of humans and animals by removing substances such as pus (purulent exudate), fibrin, coagulated blood, blood crusts and necrotic tissue. This latter type of cleaning is especially important for the treatment of wounds, burns and dermatoses, e.g. for the so called enzymatic debridement, but can also be carried out in other areas of the living body, where the contaminants can occur. The uretra and the urinary bladder are examples of such other areas.

The normal digestive effect of the enzymes in the animals, as well as their autolytic action in the animals post mortem, are not included in the term cleaning.

Technical background

In krill (which belongs to the order Euphausiaceae), a mixture of different enzymes exists, such as e.g. proteinases (with acidic and neutral-to-alkaline pH-optimun), peptidases (exo- and endopeptidases), lipases, phospholipases, amylases and other carbohydrate degrading enzymes, phosphatases, nucleases, nucleotidases and esterases (T.E. Ellingsen; Biokjemiske Studier over Antarktisk Krill; Dr. ing avhandling; Institutt for Teknisk Biokjemi, Norges Tekniske Högskole, Trondheim (1982). The proteolytic (trypsin-like) activity existing in a water extract from krill has been studied and described (C.-S. Chen et al; J. Food Biochem. 2 (1978) p. 349-66)). Different protease activities in water extracts from capelin have also been described previously (A Gildberg; Autolysis of fish tissue - General aspects; Thesis; Institute of Fisheries, University of Tromsø Norway (1982)).

As early as 1913 proposals were made to use enzymes in detergents. Enzyme compositions for cleaning of dead materials, e.g. as laundry agents, have previously been based on different microbial proteases from the genus Bacillus. One such protease commonly used is subtilisin derived from Bacillus subtilis strains and marketed among others under the name Alcalase^{R} (Novo Industri, Copenhagen, Denmark). Different lipases have also been used for cleaning, especially for enabling degradation of lipids. In addition to enzymes, such compositions have contained also different anionic, cationic and neutral detergents together with blanchophores, such as perborates. The enzymes used hitherto have, like enzymes in general, been relatively unstable.

The most important enzyme compositions on the market for debridement of the components mentioned above are Streptokinase-streptodornase (Varidase^{R}, Lederle Lab., American Cyanamid Company, Wayne, New Jersey, USA), stabilized crystalline trypsin (Trypure^{R}, Novo Industri, Copenhagen, Denmark) and bovine fibrinolysin combined with deoxyribonuclease (Elase^{R}, Parke Davis & Company, Detroit, Michigan, USA). Streptokinase acts on necrotic material mainly by its effect on DNA and streptodornase has a specific fibrinolytic effect. Trypsin acts proteolytically and is extracted from bovine pancreas. Fibrinolysin-deoxyribonuclease is a combination of two enzymes - one fibrin degrading enzyme and one acting on deoxyribonucleic acid which is an important component in pus.

One specific pepsin-like enzyme (pepsin I) from Mallotus villosus (capelin) has been proposed to be used in the medical treatment of burns (Gildberg A; cited above p. 89 - 90). The use of one specific enzyme acting on one type of substrates is likely to achieve only a limited breakdown of the contaminants present in wounds. However, no combination of this pepsinlike enzyme with other enzymes has been proposed for the therapeutical cleaning of humans.

The enzyme preparations mentioned above suffer from several drawbacks. Thus, all of them are relatively unstable leading to a rapid decrease in their activity, either during storage or use. Their activities are often limited to a certain pH-range, e.g. neutral to moderately alkaline pH. Their activities are also in many cases restricted to certain temperature intervals. At a temperature above +50 ^{o}C, a rapid loss of activity is observed and at room temperatures or normal outdoor temperatures, their activities are low.

The effect of Varidase^{R}, Trypure^{R} and Elase^{R} are relatively poor for its purpose. Only a moderate debriding effect is usually achieved after a treatment over a period of three weeks.

The objects of the invention

One object of the present invention is to provide an improved cleaning composition with respect to the drawbacks mentioned above. Another object is to provide a method for the preparation of an improved composition to be used for the removal of contaminants containing substances of biological origin or degradation products from such substances, especially contaminants containing both proteins and lipids. A third object is to provide a new and improved method for cleaning living or dead material from the contaminants mentioned above, especially for the debridement of fibrin, coagulated blood and devitalized tissue by degrading these constituents and thereby facilitating their removal without apparently increasing their water content. The invention hence is based on compositions having more effective enzymes than the prior art compositions. This is valid for the total enzymatic effect on the contaminant in question.

The invention

These objects are achieved by using a composition containing an effective amount of an enzyme preparation which is capable of dissolving contaminants of biological origin and which derives from the animals mentioned in the introductory part. Enzyme mixtures from these animals can be obtained in a high yield and in a simple way. At present the most preferred and useful sources for the enzyme preparation are animals of the order Euphausiaceae, e.g. antarctic krill (Euphausia superba), Euphausia crystallorophias, and related species and other species of krill including Meganyctiphanes norvegica, Tysanoessa inermis and other related species. Among fishes, those of

the genus Mallotus, particularly of the species Mallotus villosus (capelin), are preferred according to the invention. Among other sources, mackerel may be mentioned.

The most important enzyme activities for the invention appear to originate from the digestive tract of the animals. The complex mixture of different enzyme activities obtained from the animals is probably the explanation why the composition effects a surprisingly rapid degradation of biological contaminants irrespective of their origin. The enzymes are active in alkaline, neutral and acid medium, and can be utilized in cleaning compositions together with different surfactants (tensides and emulgators), and/or other components such as carriers and additives.

The fact that the composition may contain a mixture of enzymes makes it useful for the removal of contaminants containing mixtures of substances selected from the group consisting of lipids, phospholipids, biopolymers such as proteins, peptides, nucleic acids, mucopolysaccharides and polysaccharides, and degradation products of such compounds. These compounds are present in pus, blood crust and necrotic tissues.

According to the invention, particularly good results are obtained if the enzymes used have molecular weights within the range of 15 000 to 80 000 Daltons or active aggregates of such enzymes. Especially, enzymes having molecular weights from about 20 000 to about 40 000 Daltons are preferred. These ranges apply to enzymes obtained by a water extraction of the homogenized animals, i.e. especially to enzymes being water soluble during the extraction.

One embodiment of the invention relates to a new enzyme cleaning composition and to a method for the preparation thereof. The method is characterized in that an enzyme preparation, originating from an animal of the order

Euphausiaceae or from a fish, is mixed with, dissolved in, bound to or otherwise combined with one or more water-insoluble or water-soluble aqueous or non-aqueous carriers, if necessary together with suitable additives. The new composition may be in the form of an ointment, a powder, a paste, a cream, a spray, a gel, a liniment, a bandage, an oil, a tablet, a syrup, a granulate, a capsule, a tablet etc.

Certain non-sterile homogenous water solutions only consisting of buffer substances, water as the single solvent, and an enzyme preparation from the animals in question are excluded from the concept of the new composition. Such water solutions have been described in the publications cited above. The same applies to lyophilized water extract containing no additives.

In the presently most preferred embodiment, the enzymes in the preparation used are water-soluble and/or have molecular weights in the ranges mentioned above.

The invention also relates to a method for removing biological contaminants from living or dead material, e.g. by an enzymatic debridement. The method is characterized in that such contaminants being present on said material are contacted with an enzyme composition containing an effective amount of enzymes, e.g. a proteolytically and/or lipolytically effective amount of enzymes, originating from the animals mentioned above, whereafter the composition is removed from said material together with degraded or dissolved contaminants. In the presently most preferred embodiment, the method utilizes enzymes (including their active aggregates) having molecular weights from about 15 000 to about 80 000 Daltons or active aggregates of such enzymes. Especially, enzymes of molecular weights from about 20 000 to about 40 000 Daltons are preferred. Very great advantages are obtained for the removal of devitalized substances such as pus, blood crusts and

necrotic tissue, and fibrin or coagulated blood. This is especially valid when these substances are removed from a living tissue.

The time period required to degrade and/or dissolve the contaminants varies from case to case, but should be selected sufficiently long to allow the enzymes to degrade and/or dissolve the contaminants. The treatment can be repeated, if necessary.

The enzyme preparation used may contain a wide variety of different enzymes e.g. of the types mentioned above. According to the invention it is possible to use compositions in which one or more of these enzymes have been removed by methods known per se, e.g. by adding an inhibitor specific for one enzyme or by removing the enzyme in question.

The proteolytic activity at optimum temperature is high in the pH-range 5 - 10. The pH-optimum is in the range 7 - 9. The temperature range for good proteolytic activity is 25 - 70 ^{o}C and the optimal temperature is within the range of 30 - 55 ^{o}C. Activity is maintained down to 0 ^{o}C.

The temperature and pH-ranges given above may all be utilized for different applications of the invention, although certain potential embodiments may also utilize other ranges.

The preparation of the enzymes from the animals is performed by well known methods. Hence, fresh or freshly frozen animals may by homogenized and extracted with aqueous medium (e.g. water). The extract obtained may be lyophilized and stored. The extract can be further purified e.g. by extraction with a lipid dissolving solvent in order to extract lipids. If further purification is required, gel-, ultra- or membrane filtration may be carried out. Other purification steps, which are potentially useful, are ion

exchange and affinity chromatography. The extraction and the homogenization should be carried out in the cold below or close to +5 °C.

The enzyme preparations which are obtained by a water extraction, may be used directly or, if necessary, after further purification. In most cases, it is of advantage to lyophilize a preparation from which lipids have been extracted. In such cases, the powder obtained may be stored for a long time. Each field of use requires specific compositions or forms thereof. Such forms are known per se. Thus the invention relates to enzyme compositions of different physical forms, such as lyophilized enzymes, homogeneous aqueous solutions or the enzyme compositions mentioned above and claimed to be new. The exact amount of enzymes in each composition varies from case to case - from the pure lyophilized extract to very complex detergent compositions. The amount of enzyme should be effective in degrading and/or dissolving the contaminants intended, without giving unnecessary adverse effects on the material from which the contaminants are to be removed. The other components in the new cleaning composition should be selected so that they will not adversely affect it under the condition required for its use.

The amount of the enzyme preparation existing in the final composition may vary from 0.0001 % (w/w) up to 100 %. With respect to protease activity, the final composition may contain from 0.0001 to 0.1 enzyme units per mg. Depending on the purity of the enzyme preparation used, other ranges may apply. The enzyme units above are given as μmol tyrosine equivalents per min with casein as substrate.

In compositions according to the invention different additives and carriers may be incorporated. Suitable carriers may be sterile, aqueous and physiologically acceptable salt solutions, organic and inorganic gel-forming materials, e.g. polymeric ones, silicone oils and

other substances and mixtures thereof providing the desired physical properties of the composition. Among additives antimicrobials, perfumes, different anionic, cationic, zwitterionic and neutral surfactants (tensides and emulgators) may be mentioned. Such components are already known per se in connection with other enzyme compositions for laundring or enzymatic debridement, see e.g. German patent application 2 130 833, GB patent specifications Nos. 1 280 497, 1 296 901 and 1 573 964 and US patent specifications Nos. 3 627 688, 3 855 142, 4 212 761, 4 243 546, 4 242 219, 4 287 082, 4 305 837 and 4 307 081, which all are incorporated herein by reference.

For treatment of injured living tissues, it is advantageous to use a composition containing a physiologically and pharmaceutically acceptable carrier or no carrier at all. Such a composition may be sterile. Sterilization may be carried out e.g. by sterile filtration when the composition is a solution. Sterile compositions may also be obtained by mixing sterile components under aseptic conditions.

The different embodiments of the invention will further appear from the claims, hereby enclosed to the specification. Below, the invention will be illustrated by different non-limiting examples.

EXAMPLE 1

A. Preparation of a krill extract

Krill, Euphausia superba, caught during the Antarctic summer and frozen immediately and stored for about 2 years at about -80 ^{o}C, are placed in a room at about +5 ^{o}C. When the krill is nearly thawed, 25 g of the krill is mixed with 50 ml of deionized water having a temperature of 0 ^{o}C. The mixture is homogenized and then centrifuged in the cold (about 0 ^{o}C) for half an hour at 12 500 g. The red supernatant is decanted and saved. The sediment is re-

suspended in 50 ml deionized water and centrifuged as above. The new supernatant is decanted and combined with the supernatant from the first extraction.

In order to remove lipids from the extract 20 ml of CCl_4 is added to the combined supernatant and homogenized in the cold (0 °C). The mixture is centrifuged at 2500 g for 15 min in the cold. The water-phase is removed and extracted once more with CCl_4 and centrifuged as described above. The water-phase is used according to 1B, where it is referred to as the water extract.

B. Further purification by gel chromatography

20 ml of the water extract from A is chromatographed on Sephadex^R G-100 (dextran crosslinked with epichlorohydrin, Pharmacia Fine Chemicals AB, Uppsala, Sweden) in a column having a diameter of 3,1 cm and a height of 69 cm. The column is equillibrated and eluted (30 ml per hour) with Tris-HCl buffer (0.05M, pH 7.5) at +5 °C. Fractions are collected. The elution profile is monitored spectrophotometrically by measuring the UV adsorption at 280 nm and the proteolytic activity of the fractions are determined separately. The enzymatically active fractions are pooled and dialyzed against deionized water. Finally, the pooled fractions are lyophilized and used according to the invention.

The proteolytic activity in the fractions and in the lyophilized preparation is determind by using hemoglobin and/or casein as substrates (Rick, W I; Methods of Enzymatic analysis; Ed Bergmeyer, H U; Vol 2, p. 1013-23 (1974); Academic Press, New York). By carrying out gel chromatography, enzyme activity is mainly recovered from fractions corresponding to the molecular weights of 20 000 to 40 000 Daltons.

EXAMPLE 2

Preparation of a capelin extract

Capelin (Mallotus villosus), caught off the coast of Finnmark (Norway) in the month of September, was frozen and stored at -20 °C for one year. The frozen capelin was then placed at 5 °C. After 24 hours the intestines including the digestive tract were removed from the partially thawed capelin. 25 g of intestines were mixed with 50 ml of deionized water and homogenized at 0 °C. The mixture was subsequently centrifuged at 12500 g for half an hour. The partially cloudy supernatant is decanted and saved. The sediment is resuspended in 50 ml deionized water and centrifuged as above. The new supernatant is decanted and combined with the supernatant from the first extraction.

In order to remove lipids from the extract 20 ml of CCl_4 were added to the combined supernatant and homogenized in the cold (0 °C). The mixture was centrifuged at 2500 g for 15 min in the cold. The water-phase was removed and extracted once more with carbontetrachloride and centrifuged as described above. The water-phase was finally lyophilized.

EXAMPLE 3

Compositions containing enzymes from Mallotus villosus

A. 10 mg of the lyophilized enzyme preparation from capelin (according to example 2) are mixed with 0,4 mg calcium acetate and amylum resorb ad 1 g (Biosorb^R; Arbrook, Kirkton Campus, Livingston, Scotland).

The homogenous powder so obtained is agitated in 10 g of physiological sodium chloride solution (saline) and can be used for wet dressings.

B. 10 mg of the lyophilized capelin preparation (from example 2) are mixed with a polyethylene glyool gel (containing equal parts of Macrogol 600 and Macrogol 800 (Hoechst, Federal Republic of Germany)) ad 1 g. A 1 % enzyme composition having a semi solid, homogenous consistency is obtained.

EXAMPLE 4

Composition containing krill enzymes in a solution

10 mg of a lyophilized enzyme preparation (from example 1B) are mixed with 0.4 mg calcium acetate and amylum resorb ad 1 g. The homogenous powder so obtained is shaken with 10 g saline and the composition obtained is used in order to soak a sterile compress having the size of 3 x 3 cm and containing 4 layers of a gauze. The wet compress is applied on a necrotic wound. A gauze is wound two turns around the compress in order to affix it. This bandage is then further affixed with an adhesive plaster. The compress is soaked every fourth hour with the composition. In order to limit the application only to that part of the compress which covers the wound, the mixture is applied by the use of an injection cannula having a coarse apex. Once a day, the whole dressing is removed and replaced by a fresh dressing. During this operation degraded and disintergrated necrotic parts must be removed mechanically. In the specific treatment carried out, the wound appeared clean after 1 week.

EXAMPLE 5

Effect of krill enzymes on necrotic materials

In order to examine the effect of the krill enzymes on necrotic components existing on the skin of humans, the following experiment was carried out:
Necrotic material was applied on the intact skin of the upper side of the right hands of two persons. The necrotic material was from a wound in a leg. The material consisted

of devitalized collagen tissue, coagulated blood, fibrin, pus and crusts. The amount applied for each person was 0.5 g.

The necrotic material on each person was covered with a compress (area 10 cm^2), which had been soaked with the krill enzyme composition prepared as in example 4. Each compress was covered with a gauze. After 4 hours both of the compresses were nearly completely dry and therefore more of the enzyme composition had to be added. After 12 hours the compresses were removed and the necrotic material remaining on each hand was weighed separately. The material showed characteristic signs of degradation and had decreased their weights with about 25 %. The skin, contacted with the necrotic material during the test, were unaffected. No negative symptoms were experienced by the test persons.

EXAMPLE 6

In vivo test on a person having a minor necrotic wound A person having a minor ulcer containing necrotic material on his right forefinger, treated himself over a period of 5 days with daily application of a 1 % krill enzyme solution (prepared as in example 4). The wounded area tolerated the enzyme composition well and showed a significant tendency to become clean and to epithelialize. No adverse effects were observed.

EXAMPLE 7

The cleaning effect on lipid rich seborrhoic skin by krill enzymes combined with an amphoteric tenside

A conventional detergent was used consisting of 18,00 % (w/w) alkylimidazoline, 1,90 % (w/w) lauryl-polypeptid condensate, 1,70 % (w/w) undecylenyl-polypeptid condensate, 28,00 % (w/w) modified alkyl ether sulfate buffered with fat substances (lanoline), 1,40 % (w/w) coconut-amine

sarcosinate, 1,90 % (w/w) undecylene acid, 2,00 % (w/w) undecylene-monoethanolamide sulfosuccinate, lactic acid q.s. pH 6,4 and aqua bi dest and 100,00 % (w/w).

An amount of the lyophilized krill enzyme preparation (from example 1B) was added to the detergent giving a 1 % (w/w) enzyme detergent composition. An area of 10 cm^2 of skin in regio sternalis of two persons, having an apparent seborrhoea oleosa in this region, was selected for testing. These areas were washed twice a day during five days with the enzyme detergent composition. Samples from the same area were taken both before and after the washing period. The samples were collected and analysed photometrically according to Schaeffer H and Kuhn-Bussius H. (Arch. Klin. u. exper. Dermatol. 238 (1970) p. 429).

The result showed a significant decrease in the transmission value for both of the two persons examined. Hence, this indicates a degradation of the lipid of the skin by the enzyme composition used. Comparatively very little effect was obtained, when the two persons used only detergent in the abscence of enzymes.

EXAMPLE 8

Comparative washing experiments on textile. A comparison between enzyme from E.superba (krill), Alcalase^R and destilled water

Equal pieces (5 x 5 cm) were cut from a homogenous part of a cloth. The cloth was twofolded having one side of silk and the other of a weft consisting of a mixture of cotton and synthetic fibers. Six of the pieces were stained with blood, six with milk and six with indian ink. To each of six Ehrlenmeyer flasks, 100 ml of an aqueous solution (0.5 % w/v) of the lyophilized krill enzyme were added (the lyophilized krill enzyme was obtained from example 1B). To each of six other Ehrlenmeyer flasks, 100 ml of a 0.5 % (w/v) of Alcalase^R (Novo Industri, Copenhagen, Denmark)

solution in aq. dest were added. 100 ml aq. dest were added to six other Ehrlenmayer flasks.

To each of two flasks containing the krill enzyme solution, to each of two flasks containing the Alcalase^{R} solution and to each of two flasks containing aq. dest, one of the pieces stained with blood was added. Analogously, the other stained pieces were added to the remaining flasks, so that only one piece was present in each flask. This meant that each of the compositions were allowed to act in duplicate on cloth stained with blood, milk and indian ink. Thereafter, the flasks were agitated on a shaking water bath for one hour at +45 ^{o}C. After this treatment, the washing liquid was decanted and 100 ml aq. dest were added to each flasks. The flasks were then sealed with a rubber plug and agitated heavily for one minute. Thereafter the pieces of cloth were collected and washed slowly in flowing tap water for 10 minutes. The pieces were folded in clean white towels and allowed to dry.

The effect of the washing, i.e. the brightness of the cloth, was then determined by a double blind test, i.e. the examiner did not know which washing composition each piece had been treated with. The washing effect, i.e. the brightness and cleanness of the cloth, was evaluated visually in the order of precedence; 0 = completely clean cloth; 1 = insignificant amounts of remaining dirt; 2 = moderate amount of remaining dirt; 3 = considerable amounts of remaining dirt. The results from the washings are presented in Table 1.

Table 1

The washing effect of 0.5 % (w/v) krill enzyme in aq. dest; 0.5 % (w/v) Alcalase^{R} in aq. dest; and aq. dest without addition of enzymes.

Contaminants	0.5 % krill-enzyme in aq. d.		0.5 % Alcalase^{R} in aq. d.		aq. dest alone	
	Cloth I	Cloth II	Cloth I	Cloth II	Cloth I	Cloth II
Blood	1	1	2	2	2	3
Milk	1	1	2	1	2	2
Indian ink	2	2	2	2	3	3

From Table 1 it is seen that the enzyme composition from krill is more effective than Alcalase^{R} or aq. dest. Similar tests can be performed with an analogous composition containing capelin enzymes.

EXAMPLE 9

Degradation of fibrin, necroses and coagulated blood

A. Fibrin

Fibrin was dissected into pieces varying in weight from 0.2 - 0.3 g. For each enzyme composition used, 20 test tubes were numbered and to each of them one piece of preweighed fibrin was added. To the 20 tubes of each composition, equal amounts of the enzyme composition were added. Varidase^{R} was used in the dilution of 1:20 (w/v based on the commercial preparation) in destilled water. Trypure^{R} was used in the concentration of 1:15 (w/v based on the commercial preparation) in saline. The preparations of the other enzymes used were diluted in saline and the concentrations of the composition so obtained are given in Table 2. The krill enzymes and capelin enzymes used were the lyophilized preparations obtained according to example 1B and 2, respectively. The study was performed at a temperature of +33 ^{o}C which is the same as the temperature in wounds. After 12 hours and 24 hours, respectively, the reactions were stopped

and the fibrin remaining was collected and kept on a wet filter paper for 60 seconds and weighed. Papain, Ficin and Pankreatin were all obtained from E. Merck, Darmstadt, Germany.

Table 2

The influence of different enzyme compositions on fibrin. Concentrations are given in % w/v.

\- denotes 0 - 25 % decrease in weight
-- denotes 26 - 50 % decrease in weight
--- denotes 51 - 75 % decrease in weight
---- denotes 76 - 100 % decrease in weight

Enzyme	Reading after 12 hour	24 hour
Trypure®	----	nothing left
Varidase®	--	--
Ficin 1 %	-	-
Papain 1 %	--	--
Pankreatin® 1 %	---	---
Krill enzymes 1 %	----	nothing left
Capelin enzymes 1 %	----	nothing left

Fibrin was dissolved in the shortest time by the compositions containing krill enzymes, capelin enzymes and Trypure® and in the order given. Varidase® and Alcalase® were similar to ficin, and papain had the weakest effect.

B. Degradation of necroses

Necroses were dissected in pieces of 0.2 - 0.3 g. For each enzyme composition used, 20 test tubes were numbered and to each of these tubes one piece of the preweighed necroses were added. To the 20 test tubes of each composition, equal amounts of each enzyme composition were added. The compositions used are

given in Table 3 and their concentrations are given as % (w/v) in saline. Varidase^{R} and Trypure^{R} were diluted according to example 8A. After time intervals of 12, 24, 36 and 72 hours, the degradation was stopped and the necroses were collected and weighed.

Table 3

The influence of different enzyme compositions on necroses. Concentrations in % are calculated on a w/v basis.

----	decrease in weight	76 - 100 %
---	decrease in weight	51 - 75 %
--	decrease in weight	26 - 50 %
-	decrease in weight	1 - 25 %
0	status quo	0 %
+	increase in weight	1 - 25 %
++	increase in weight	25 - 50 %
+++	increase in weight	51 - 75 %
++++	increase in weight	75 - 100 %

Enzyme composition		Reading after			
	Conc.	12 hrs	24 hrs	36 hrs	72 hrs
Varidase®	1:20 (w/v)	+++	+++		
Trypure®	1:15 (w/v)	++	++		
Ficin	0.5 %	0	0	0	-
	1.0 %	+	0	--	---
	2.0 %	+	-	-	--
Papain	0.5 %	0	+		+
	1.0 %	0	+		+
	2.0 %	+	+	+	+
Alcalase®	0.5 %	0	-	0	-
	1.0 %	-	-	--	--
	2.0 %	-	-	-	-
Krill enzymes	0.5 %	-	--	--	--
	1.0 %	--	---	---	---
	2.0 %	-	---	----	----
Capelin	0.5 %	--	--	---	--
enzymes	2.0 %	-	--	--	---
Pankreatin®	0.5 %	0	++	++	++
	1.0 %	+	+++	+++	+++
	2.0 %	++	++++	+++	+++

Apparently, Alcalase^R, krill enzymes and capelin enzymes are really able to dissolve necroses and do not increase the water content thereof like Varidase^R, Trypure^R, and saline. However, when regarding their influence on the necroses, papain and Pankreatin^R show the same tendencies as the compositions on the market, i.e. an increase in weight due to an increase in water content. Ficin has an effect similar to but weaker than Alcalase^R, the krill enzymes and the capelin enzymes.

C. Degradation of coagulated blood

Coagulated blood was dissected and portioned into test tubes arranged as in example 9A and B. The enzyme preparations used were dissolved in saline, and the concentrations (% w/v) of the compositions so obtained are given in Table 4. After the time intervals 12 and 24 hours, respectively, the degradation was stopped, and the blood coagels were collected and weighed.

Table 4

The influence of different enzyme compositions on coagulated blood.

-	decrease in weight	0 - 25 %
--	decrease in weight	26 - 50 %
---	decrease in weight	51 - 75 %
----	decrease in weight	76 - 100 %

Enzyme composition	conc.	Reading after 12 hrs	48 hrs
Varidase^R	1:20 (w/v)	-	--
Saline	0.9 %	-	-
Ficin	1.0 %	-	--
Papain	1.0 %	-	-
Alcalase^R	1 %	-	-
Pankreatin^R	1.0 %	--	--
Krill enzymes	1.0 %	---	----
Capelin enzymes	1.0 %	---	---

From Table 4 it is seen that for coagulated blood, the krill enzymes were superior to the capelin enzymes, which both showed an effect superior to the other enzyme compositions investigated.

EXAMPLE 10

Studies of different enzyme compositions on healthy skin

Different solutions of ficin, papain. Alcalase^{R}, Pankreatin^{R}, krill enzymes and capelin enzymes - all at concentrations of 1 ½ % (w/v) - were applied (0.3 ml) on the healthy skin of two experimental persons and covered by a thin bandage. No sign of inflammation could be observed on the skin after 12 hours. The same observation was found when using Varidase^{R} and Trypure^{R}. Hence, the enzymes apparently only attack necrotic tissues in the concentration ranges used in the tests.

CLAIMS

1. Enzyme composition, containing an effective amount of an enzyme preparation which degrades contaminants of biological origin and which derives from an aquatic animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the fishes preferably of the genus Mallotus, for use as a therapeutical and/or non-therapeutical cleaning agent, provided that pepsin I from Mallotus villosus is comprised only when used in combination with other enzymes and with regard to the therapeutical cleaning of mammals.

2. Enzyme composition according to claim 1, c h a r a c t e r i z e d in that the enzymes of the composition have molecular weights from about 15 000 to about 80 000 Daltons or active aggregates of such enzymes.

3. Enzyme composition according to claim 1 or 2, c h a r a c t e r i z e d in that it is capable of dissolving and removing contaminants containing mixtures of substances selected from the group of lipids, phospholipids, proteins, peptides, nucleic acids, polysaccharides, mucopolysaccharides and degradation products of these substances.

4. Enzyme composition according to claim 3, c h a r a c t e r i z e d in that it is capable of removing from living tissues, devitalized components, such as necrotic tissues, and pus, and fibrin and coagulated blood.

5. Enzyme composition according to claim 2, c h a r a c t e r i z e d in that the enzyme preparation is combined with a carrier and/or an additive.

6. Enzyme composition according to claim 5, c h a r a c t e r i z e d in that it is in a form selected from the group consisting of ointments, creams, sprays, powders, pastes, gels, liniments, bandages, oils, tablets, capsules, syrups, solutions.

7. Enzyme composition according to claim 1, c h a r a c t e r i z e d in that it contains one or more surfactants.

8. Method for the preparation of an enzyme composition to be used as a therapeutical and/or non-therapeutical cleaning agent, c h a r a c t e r i z e d in that an enzyme preparation deriving from an animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the fishes, preferably of the genus Mallotus, is combined with a carrier.

9. Method for the therapeutical and non-therapeutical cleaning by removing biological contaminants from living or dead material, c h a r a c t e r i z e d in that such contaminants being present on said material are contacted with an enzyme composition, containing an effective amount of an enzyme preparation originating from an aquatic animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the fishes, preferably of the genus Mallotus, and that, after a sufficient amount of time has elapsed, the composition is removed from said material together with degraded and dissolved contaminants, provided that pepsin I from Mallotus villosus is comprised only when used in combination with other enzymes and with regard to the therapeutical cleaning of mammals.

10. Method according to claim 9, c h a r a c t e r - i z e d in that the enzymes used for the preparation of the composition have molecular weights in the range of 15 000 - 80 000 Daltons or active aggregates thereof.

11. Method according to claim 10, c h a r a c t e r - i z e d in that the enzyme composition is capable of removing contaminants containing mixtures of substances selected from the group consisting of lipids, phospholipids, proteins, peptides, nucleic acids, polysaccharides, mucopolysaccharides or degradation products of these substances.

12. Method according to claim 11, c h a r a c t e r - i z e d in that the contaminants are devitalized material, such as necroses and pus, or fibrin, coagulated blood or blood crusts.

13. Method according to claim 12, c h a r a c t e r - i z e d in that the contaminants are present on or in a living tissue and that the removal is an enzymatic debridement.

14. Method according to claim 10, c h a r a c t e r - i z e d in that the contaminants are removed from dead material, e.g. in the laundring of a textile.

15. Cleaning composition whenever prepared according to claim 8.

AMENDED CLAIMS

[received by the International Bureau on 02 April 1984 (02.04.84); original claims 1, 8 and 9 amended; claims 2 – 7 and 10 – 15 unchanged]

1. Enzyme composition, containing an effective amount of an enzyme preparation which degrades contaminants of biological origin and which derives from an aquatic animal selected from the group consisting of animals belonging to the order Euphausiaçeae or to the genus Mallotus, for use as a therapeutical and/or non-therapeutical cleaning agent, provided that pepsin I from Mallotus villosus is comprised only when used in combination with other enzymes and with regard to the therapeutical cleaning of mammals.

2. Enzyme composition according to claim 1, c h a r a c t e r i z e d in that the enzymes of the composition have molecular weights from about 15 000 to about 80 000 Daltons or active aggregates of such enzymes.

3. Enzyme composition according to claim 1 or 2, c h a r a c t e r i z e d in that it is capable of dissolving and removing contaminants containing mixtures of substances selected from the group of lipids, phospholipids, proteins, peptides, nucleic acids, polysaccharides, mucopolysaccharides and degradation products of these substances.

4. Enzyme composition according to claim 3, c h a r a c t e r i z e d in that it is capable of removing from living tissues, devitalized components, such as necrotic tissues, and pus, and fibrin and coagulated blood.

5. Enzyme composition according to claim 2, c h a r a c t e r i z e d in that the enzyme preparation is combined with a carrier and/or an additive.

6. Enzyme composition according to claim 5, c h a r a c t e r i z e d in that it is in a form selected from the group consisting of ointments, creams, sprays, powders, pastes, gels, liniments, bandages, oils, tablets, capsules, syrups, solutions.

7. Enzyme composition according to claim 1, c h a r a c t e r i z e d in that it contains one or more surfactants.

8. Method for the preparation of an enzyme composition to be used as a therapeutical and/or non-therapeutical cleaning agent, c h a r a c t e r i z e d in that an enzyme preparation deriving from an animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the genus Mallotus, is combined with a carrier.

9. Method for the therapeutical and non-therapeutical cleaning by removing biological contaminants from living or dead material, c h a r a c t e r i z e d in that such contaminants being present on said material are contacted with an enzyme composition, containing an effective amount of an enzyme preparation originating from an aquatic animal selected from the group consisting of animals belonging to the order Euphausiaceae or to the genus Mallotus, and that, after a sufficient amount of time has elapsed, the composition is removed from said material together with degraded and dissolved contaminants, provided that pepsin I from Mallotus villosus is comprised only when used in combination with other enzymes and with regard to the therapeutical cleaning of mammals.

10. Method according to claim 9, c h a r a c t e r - i z e d in that the enzymes used for the preparation of the composition have molecular weights in the range of 15 000 - 80 000 Daltons or active aggregates thereof.

11. Method according to claim 10, c h a r a c t e r - i z e d in that the enzyme composition is capable of removing contaminants containing mixtures of substances selected from the group consisting of lipids, phospholipids, proteins, peptides, nucleic acids, polysaccharides, mucopolysaccharides or degradation products of these substances.

12. Method according to claim 11, c h a r a c t e r - i z e d in that the contaminants are devitalized material, such as necroses and pus, or fibrin, coagulated blood or blood crusts.

13. Method according to claim 12, c h a r a c t e r - i z e d in that the contaminants are present on or in a living tissue and that the removal is an enzymatic debridement.

14. Method according to claim 10, c h a r a c t e r - i z e d in that the contaminants are removed from dead material, e.g. in the laundring of a textile.

15. Cleaning composition whenever prepared according to claim 8.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE83/00359

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

A 61 K 37/54, 37/62 C 11 D 3/386, 7/42 C 12 N 9/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 2,3	A 61 K 37/48, 37/54, 37/547, 37/62, 35/56, 35/60 C 11 D 3/386, 7/42 C 12 N 9/00
IPC 1	A 61 K 19/00, 3/46, 3/52 .../...

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [15] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X, Y	SE, C, 140 568 (E LEVIN) 2 June 1953, see inter alia Example 9 and page 5, the first paragraph	1-12,14-15
Y	DE, B2, 2 022 064 (WITCO CHEMICAL CORP) 2 December 1971, see inter alia column 3, line 50- column 4, line 16 & US 3697451 NL 7006468 BE 751007 FR 2087745 GB 1293613	1-12,14-15
X, Y	FR, A, 1 015 566 (HEVIFERM-LABRATORIUM GMBH) 15 October 1952, see inter alia page 1, the first paragraph	1-12,14-15
X, Y	GB, A, 284 668 (ALFRED EHRENREICH) 1929, see inter alia page 2, lines 45-57	1-12,14-15
X, Y	GB, A, 368 888 (H TH BÖHME AG) 1932 .../...	1-12,14-15

* Special categories of cited documents: [16]

"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

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
1984-01-25	1984-02-06
International Searching Authority [1]	Signature of Authorized Officer [20]
Swedish Patent Office	Martin Hjälmdahl

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X,Y	GB, A, 377 128 (H TH BÖHME AG) 1932, see inter alia page 2, the first paragraph	1-12,14-15
Y	US, A, 3 003 917 (THE NATIONAL DRUG CO) 10 October 1961, see inter alia column 1, lines 27-35	1-12,14-15
Y	US, A, 3 409 719 (BAXTER LABORATORIES INC) 5 November 1968, see inter alia column 1	1-12,14-15
X,Y	Journal of Food Biochemistry, Vol 2, issued 1978 (Westport, Connecticut), C-S Chen et al "Purification and properties of trypsin-like enzymes and a carboxypeptidase A from Euphausia superba", see pages 349-366	1-12,14-15
X,Y	A Gildberg " Autolysis of fish tissue-general aspects", Thesis, published June 1982, by Institute of fisheries, University of Tromsö (Tromsö, Norway), see pages 75-90 especially pages89-90	1-12,14-15
X,Y	Chemical Abstracts Vol 89 (1978), abstract No 55330a, Biologia (Bratislava) 1978, 33 (6), 485-95 (Eng)	1-12,14-15
X,Y	Chemical Abstracts Vol 96(1982), abstract No 176748w, Agric Biol Chem 1982, 46 (3), 691-6 (Eng)	
X,Y	Chemical Abstracts Vol 96 (1982), abstract No 178255g, J Food Biochem 1981, 5(1), 63-8 (Eng)	1-12,14-15

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

II Fields Searched (cont)

National Cl 30h: 2/04
US Cl 424: 94; 252: 174.12

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

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

1. [X] Claim numbers *..., because they relate to subject matter [12] not required to be searched by this Authority, namely:

*) 9-12 (to the extent that they concern therapeutical cleaning) and claim number 13

Methods for treatment of the human or animal body by therapy

2. [ ] Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

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

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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(71) Applicant: **NIPPON OIL CO. LTD.**
Minato-ku Tokyo (JP)

(72) Inventors:
- **Kitaoka, Mitomitsu**
  Yokohama, Kanagawa (JP)
- **Tsubokura, Akira**
  Yokohama, Kanagawa (JP)
- **Kiyota, Takashi**
  Yokohama, Kanagawa (JP)

(74) Representative: **Hallybone, Huw George et al**
CARPMAELS AND RANSFORD
43 Bloomsbury Square
London WC1A 2RA (GB)

(54) Method for purifying xanthophyll compound

(57) A method for purifying a xanthophyll compound involves the steps of: (a) adding a nonpolar solvent and water to an aqueous organic solvent solution containing the xanthophyll compound; (b) subjecting to liquid-liquid extraction to thereby obtain a nonpolar solvent phase; and (c) subjecting the nonpolar solvent phase to at least one step selected from the following steps (c-1) to (c-3): step (c-1) of concentrating the nonpolar solvent phase; step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction; and step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase whereby the xanthophyll compound is precipitated.

EP 0 732 378 A2

Description

BACKGROUND OF THE INVENTION

This invention relates to a method for purifying a xanthophyll compound in which impurities other than the xanthophyll compound are removed from an aqueous organic solvent solution containing the xanthophyll compound for purifying the xanthophyll compound to high purity.

The xanthophyll compound is a generic name of carotenoid compounds containing oxygen atoms in the molecule thereof, and is a red-hued or yellow-hued pigment widely distributed in microorganisms or algae, plant or animal tissues or organs. Recently, the usage for the xanthophyll compound is increasing in the field of food additives as colorants for food or beverage or as colorants for fish meat such as salmon or trout, skin parts of the fish or eggs of fowl, such as hens. In addition, the xanthophyll compound is known to have an antioxidation action and its usage as an antioxidant is promising. It has also been found that certain xanthophyll compounds exhibit cancer-prohibiting properties such that they may be expected in future to be employed as pharmaceuticals.

Of the xanthophyll compounds, astaxanthin, canthaxanthin and zeaxanthin are produced industrially by a synthetic method (Pure and Applied Chemistry, 63(1), 35-44 (1991)) and are employed as feed additives for coloration. Recently, use of a synthesized product as food or feed additive is becoming more and more difficult in consideration of safety. Thus, in conjunction with the general preference for natural products, a strong demand has been raised for technology of producing natural carotenoid compounds in substitution for synthesized products.

A number of reports have been made in connection with the technique of extracting xanthophyll compounds from natural products. For example, the method of extracting the compounds with aqueous organic solvents, such as acetone, tetrahydrofuran, dioxane, pyridine or cyclohexanone, is thought to be effective. However, if the xanthophyll compounds are extracted from microorganisms, algae or the tissues or organs of plants or animals using these solvents, polar lipid, neutral lipid or carotenoid compounds other than the xanthophyll compounds are co-extracted as impurities. Although these impurities may be removed by chromatography, this method is economically disadvantageous since a large quantity of solvents needs to be employed. Thus, an economically meritorious method capable of removing these impurities efficiently has been desired.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for purifying a xanthophyll compound whereby the xanthophyll compound may be purified and isolated easily and efficiently by removing impurities from natural products.

The above and other objects of the invention will become apparent from the following description.

According to the present invention, there is provided a method for purifying a xanthophyll compound comprising the steps of (a) adding a nonpolar solvent and water to an aqueous organic solvent solution containing the xanthophyll compound; (b) subjecting to liquid-liquid extraction to thereby obtain a nonpolar solvent phase; and (c) subjecting the nonpolar solvent phase to at least one step selected from the group consisting of the following steps (c-1) to (c-3): step (c-1) of concentrating the nonpolar solvent phase; step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction; and step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase whereby the xanthophyll compound is precipitated.

PREFERRED EMBODIMENTS OF THE INVENTION

The present invention will be explained in further detail hereinbelow.

In the purifying method of the present invention, an aqueous organic solvent solution containing a xanthophyll compound is employed as a starting material in step (a). Although there is no limitation to the aqueous organic solvent solution containing the xanthophyll compound, it may preferably be produced by dissolving a material containing the xanthophyll compound in an aqueous organic solvent, or by extracting a material already extracted and containing the xanthophyll compound with an aqueous organic solvent. More preferably, it may be produced by extracting a natural material containing the xanthophyll compound, such as microorganisms, algae or the tissues or organs of plants or animals, with an aqueous organic solvent.

Although there is no particular limitation to the aqueous organic solvent, it may preferably be enumerated by acetone, tetrahydrofuran, dioxane, pyridine, cyclohexanone or mixtures thereof thought to be effective for extracting the xanthophyll compounds.

In the purifying method of the present invention, a nonpolar solvent and water are added to the aqueous organic solvent solution containing the xanthophyll compound in step (a) and liquid-liquid extraction is carried out in step (b) to obtain a nonpolar solvent phase.

Although there is no limitation to the nonpolar solvent, it may preferably be enumerated by hexane, heptane, octane, cyclohexane, benzene, toluene, xylene, petroleum ether, naphtha, kerosene and n-paraffin. Most preferred of these nonpolar solvents are hexane, cyclohexane and petroleum ether. These solvents may be used alon or in combination.

The water is not limited to pure water and preferably may be enumerated by distilled water, ion-exchanged water, city water, industrial water, aqueous solutions of

salts, such as an aqueous sodium hydroxide solution or an aqueous sodium chloride solution, e.g. brine in which table salt, sodium chloride or sodium hydroxide is added to the aforementioned water, and sea water.

Although there is no particular limitation to the amounts of the nonpolar solvent and water added to the aqueous organic solvent solution containing the xanthophyll compound, the amount of the nonpolar solvent is preferably 0.1 to 20 parts by volume and more preferably 0.2 to 10 parts by volume to 1 part by volume of the aqueous organic solvent solution containing the xanthophyll compound, while the amount of water is usually not less than 0.1 part by volume, e.g. 0.1 to 30 parts by weight and preferably 0.2 to 10 parts by volume to 1 part by volume of the aqueous organic solvent solution containing the xanthophyll compound.

There is no particular limitation to the liquid-liquid extraction in step (b) provided the nonpolar solvent phase may thereby be obtained. Thus, it is possible to use any desired method for liquid-liquid extraction. For example, the nonpolar solvent phase may be produced by a batch type method for liquid-liquid extraction consisting of agitating a liquid mixture composed of the aqueous organic solvent solution containing the xanthophyll compound, the nonpolar solvent and the water in an agitating vessel, or by continuous liquid-liquid extraction using a mixer-settler extractor. There is no particular limitation to the extraction temperature, which may preferably be 0°C to 100°C and more preferably 10°C to 80°C, while there is also no limitation to the liquid-liquid extraction time duration, which may preferably be 10 seconds to 10 hours.

With the purifying method of the present invention, at least one of the step (c-1) of concentrating the nonpolar solvent phase; step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction; and step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase, is carried out in the step (c) for precipitating the xanthophyll compound.

The above step (c-1) may be carried out by heating at atmospheric pressure or reduced pressure using a rotary evaporator or the like for distilling off part or all of the solvent in the nonpolar solvent phase. Although there is no particular limitation to the factor of concentration, it is preferably 1.5 to 1,000 folds. By this step (c-1), the nonpolar solvent phase is concentrated for selectively precipitating only the xanthophyll compound.

The above step (c-2) may be carried out by a method consisting of adding water to the nonpolar solvent phase and carrying out liquid-liquid extraction in accordance with a batch system as in the case of the above-mentioned liquid-liquid extraction. Alternatively, the step (c-2) may be carried out by continuous liquid-liquid extraction using a mixer-settler extractor. The water added is not limited to pure water and the aqueous solutions as well as the water enumerated for the step (a) may also be employed. There is no limitation to the amount of water added for the step (c-2) and usually may be not less than 0.1 part by volume and, for example 0.1 to 30 parts by volume and preferably 0.2 to 10 parts by volume to 1 part by volume of the nonpolar solvent phase. There is also no limitation on the temperature for liquid-liquid extraction which may preferably be 0°C to 100°C and more preferably 10°C to 80°C. Although there is also no particular limitation to the time of liquid-liquid extraction, it is preferably 10 seconds to 10 hours. The liquid-liquid extraction with water may be repeated plural times. By lowering the aqueous organic solvent concentration in step (c-2), only the xanthophyll compound may be precipitated selectively.

In the step (c-3), the xanthophyll compound is precipitated by simply adding a nonpolar solvent to the nonpolar solvent phase. The nonpolar solvent enumerated above for the step (a) may be used as the nonpolar solvent for the step (c-3). That is, the same or different nonpolar solvent may be used for the step (c-3). Although no limitations are imposed on the amount of the nonpolar solvent added, it is usually not less than 0.1 part by volume, typically 0.1 to 30 parts by volume and preferably 0.2 to 10 parts by volume to one part by volume of the nonpolar solvent phase.

Although the xanthophyll compound may be precipitated efficiently and with high purity by carrying out one of the steps (c-1) to (c-3) alone, these steps may be suitably combined with one another, if so desired.

The xanthophyll compound precipitated by at least one of the steps (c-1) to (c-3) may be recovered by any suitable method. There is no particular limitation to the recovering method which may usually be carried out by filtration or centrifugal separation.

With the purifying method according to the present invention, carotenoid compounds other than the xanthophyll compound may be removed from the aqueous organic solvent solution containing the xanthophyll compound in addition to the impurities other than the carotenoid compounds. It should be noted that the carotenoid compounds other than the xanthophyll compound were heretofore difficult to separate and remove from the aqueous organic solvent solution containing the xanthophyll compound.

There is no particular limitation to the xanthophyll compound that may be purified in accordance with the purifying method of the present invention, provided that the compound is a carotenoid compound containing an oxygen atom in the molecules thereof. Examples of the xanthophyll compound includes astaxanthin, canthaxanthin, zeaxanthin, adonixanthin, adonirubin, β-cryptoxanthin, echinenone, asteroidenone, 3-hydroxyechinenone, rhodoxanthin, fucoxanthin, lutein, capsanthin and capsorubin.

With the purifying method of the present invention, the xanthophyll compound, which cannot be purified with the conventional technique, can be purified easily and efficiently. In particular, the purifying method of the present invention may be usefully applied to purification

from a material derived from natural products. The purified xanthophyll compounds may be applied to a variety of usages, including food additives, feed additives, starting materials therefor, or starting materials for pharmaceuticals.

EXAMPLES OF THE INVENTION

The present invention will be explained with reference to Preparation Examples and Examples, which are merely illustrative and are not intended for limiting the scope of the invention.

An E-396 strain employed in the Preparation Examples 1 and 2, which was identified as not belonging to any known genus, was deposited at the NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN TECHNOLOGY, AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY on April 27, 1993 and has been accorded accession number FERM BP-4283. FERM BP-4283 has been accepted for deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. All restrictions on the availability to the public of FERM BP-4283 will be irrevocably removed upon the granting of a patent.

Preparation Example 1

Preparation of Acetone-Extracted Colored Liquid

To 100 g (wet weight) of an E-396 cultured strain (FERM BP-4283), which was bacteria producing astaxanthin, adonixanthin, adonirubin, canthaxanthin, and echinenone, was added 1 liter of acetone. The resulting mass was agitated for one hour and filtered to produce an acetone-extracted colored liquid (A). The xanthophyll compound concentration in the colored liquid (A) was 80 ppm. The purity of the xanthophyll compound in the solid content contained in the colored liquid (A) was 9.7%. The colored liquid (A) contained β-carotene, phospholipid and neutral lipid as impurities.

Preparation Example 2

Preparation of Tetrahydrofuran (THF)-Extracted Colored Liquid

To 250 g (wet weight) of an E-396 cultured strain (FERM BP-4283), which was bacteria producing astaxanthin, adonixanthin, adonirubin, canthaxanthin, and echinenone, was added 1 liter of tetrahydrofuran (THF). The resulting mass was agitated for one hour and filtered to produce a THF-extracted colored liquid (B). The xanthophyll-compound concentration in the colored liquid (B) was 350 ppm. The purity of the xanthophyll compound in the solid content contained in the colored liquid (B) was 11.2%. The colored liquid (B) contained β-carotene, phospholipid and neutral lipid as impurities.

Example 1-1

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1,. were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 20 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.9 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 1-2

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 100 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 10.8 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-3

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 218 ml of an upper hexane phase. The hexane phase thus obtained was concentrated under vacuum to 20 ml using a rotary evaporator. The resulting concentrated liquid was allowed to stand at 4°C for 12 hours and the precipitate was recovered by filtration. As a result, 12.3 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in th powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 1-4

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chlorid and agitated for one hour. The resulting solution was allowed to stand for sampling 221 ml of a petroleum ether phase as an upper

phase. The produced petroleum ether phase was concentrated under vacuum up to 20 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.9 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-5

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 226 ml of a cyclohexane phase as an upper phase. The produced cyclohexane phase was concentrated under vacuum up to 20 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 12.4 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 97%.

Example 1-6

To 200 ml of the acetone-extraoted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 235 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 20 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 12.6 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-7

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 10 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 55 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-8

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 20 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 52 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-9

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 301 ml of an upper hexane phase. The produced hexane phase was concentrated under vacuum up to 10 ml using a rotary evaporator. The concentrated liquid was allowed to stand at 4°C for 12 hours and the precipitate was recovered by filtration. As a result, 57 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 100%.

Example 1-10

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 316 ml of a petroleum ether phase as an upper phase. The produced petroleum ether phase was concentrated under vacuum up to 10 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 58 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 1-11

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The

resulting solution was allowed to stand for sampling 305 ml of a cyclohexane phase as an upper phase. The produced cyclohexane phase was concentrated under vacuum up to 10 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 59 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 1-12

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 325 ml of a hexane phase as an upper phase. The produced hexane phase was concentrated under vacuum up to 10 ml using a rotary evaporator. The concentrated solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 52 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-1

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. To the produced hexane phase were added 200 ml of a 1 wt% aqueous solution of sodium chloride and the resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.2 mg of dark purplish powders were obtained. The purity of the xanthophyll compound, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-2

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. To the produced hexan phase were added 400 ml of a 1 wt% aqueous solution of sodium chloride and th resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitat was recovered by filtration. As a result, 11.8 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 2-3

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 218 ml of a hexane phase as an upper phase. The resulting hexane phase was admixed with 200 ml of city water and the resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.4 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 2-4

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 221 ml of a petroleum ether phase as an upper phase. The resulting petroleum ether phase was admixed with 200 ml of city water and the resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.6 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-5

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 226 ml of a cyclohexane phase as an upper phase. The resulting cyclohexane phase was admixed with 200 ml of a 1 wt% aqueous solution of sodium chloride and the resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.9 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 97%.

Example 2-6

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 235 ml of a hexane phase as an upper phase. The resulting hexane phase was admixed with 200 ml of city water and the resulting solution was agitated for one hour. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.5 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 97%.

Example 2-7

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. The resulting hexane phase was further liquid-liquid extracted thrice with 200 ml of a 1 wt% aqueous solution of sodium chloride. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 55 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-8

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. The resulting hexane phase was further liquid-liquid extracted thrice with 100 ml of a 1 wt% aqueous solution of sodium chloride. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 50 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 2-9

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 301 ml of a hexane phase as an upper phase. The resulting hexane phase was further liquid-liquid extracted thrice with 200 ml of city water. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 59 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-10

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 316 ml of a petroleum ether phase as an upper phase. The resulting petroleum ether phase was further liquid-liquid extracted thrice with 200 ml of a 1 wt% aqueous solution of sodium chloride. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 56 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 2-11

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium hydroxide and agitated for one hour. The resulting solution was allowed to stand for sampling 305 ml of a cyclohexane phase as an upper phase. The resulting cyclohexane phase was further liquid-liquid extracted thrice with 200 ml of a 1 wt% aqueous solution of sodium chloride. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 60 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 2-12

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 325 ml of a hexane phase as an upper phase. The resulting hexane phase was further liquid-liquid extracted thrice with 100 ml of a 1 wt% aqueous solution of sodium chloride. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 53 mg of dark purplish powders

were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 3-1

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 400 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.0 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 3-2

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 223 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 400 ml of petroleum ether. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.1 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 3-3

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 218 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 400 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.6 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 3-4

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 221 ml of a petroleum ether phase as an upper phase. To the resulting petroleum ether phase were added 400 ml of petroleum ether. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.3 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 3-5

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 226 ml of the cyclohexane phase as an upper phase. To the resulting cyclohexane phase were added 400 ml of cyclohexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 12.0 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 3-6

To 200 ml of the acetone-extracted colored liquid (A) prepared in the Preparation Example 1, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 235 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 600 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 11.5 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 3-7

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 800 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 53 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chro-

matography, was found to be 98%.

Example 3-8

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 312 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 800 ml of petroleum ether. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 54 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 98%.

Example 3-9

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 200 ml of city water and agitated for one hour. The resulting solution was allowed to stand for sampling 301 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 800 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 56 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 97%.

Example 3-10

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of petroleum ether and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 316 ml of a petroleum ether phase as an upper phase. To the resulting petroleum ether phase were added 800 ml of petroleum ether. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 57 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

Example 3-11

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of cyclohexane and 200 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 305 ml of a cyclohexane phase as an upper phase. To the resulting cyclohexane phase were added 800 ml of cyclohexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 59 mg of dark purplish powders were obtained. The purity of a xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 100%.

Example 3-12

To 200 ml of the THF-extracted colored liquid (B) prepared in the Preparation Example 2, were added 200 ml of hexane and 100 ml of a 1 wt% aqueous solution of sodium chloride and agitated for one hour. The resulting solution was allowed to stand for sampling 325 ml of a hexane phase as an upper phase. To the resulting hexane phase were added 1200 ml of hexane. The resulting solution was allowed to stand at 4°C for 12 hours and the resulting precipitate was recovered by filtration. As a result, 61 mg of dark purplish powders were obtained. The purity of the xanthophyll compound in the powders, as confirmed by high performance liquid chromatography, was found to be 99%.

It is seen from the above Examples that any of the step (c-1) of concentrating the nonpolar solvent phase, the step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction and the step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase is effective in purifying the xanthophyll compound and that any suitable combination of these steps (c-1) to (c-3) also gives high purity xanthophyll compounds.

Claims

1. A method for purifying a xanthophyll compound comprising the steps of:

   (a) adding a nonpolar solvent and water to an aqueous organic solvent solution containing the xanthophyll compound;
   (b) subjecting to liquid-liquid extraction to thereby obtain a nonpolar solvent phase; and
   (c) subjecting the nonpolar solvent phase to at least one step selected from the following steps (c-1) to (c-3):

   step (c-1) of concentrating the nonpolar solvent phase;
   step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction; and
   step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase

whereby said xanthophyll compound is precipitated.

2. The method as claimed in claim 1 wherein said nonpolar solvent in said step (a) is selected from hexane, heptane, octane, cyclohexane, benzene, toluene, xylene, petroleum ether, naphtha, kerosene, n-paraffin and mixtures thereof.

3. The method as claimed in claim 1 wherein said water in said step (a) is selected from distilled water, ion-exchanged water, city water, industrial water, an aqueous solution of sodium hydroxide, an aqueous solution of sodium chloride, sea water and mixtures thereof.

4. The method as claimed in claim 1 wherein 0.1 to 20 parts by volume of the nonpolar solvent and not less than 0.1 part by volume of said water are added in said step (a) to 1 part by volume of said aqueous organic solvent solution containing the xanthophyll compound.

5. The method as claimed in claim 1 wherein said water in said step (c-2) is selected from distilled water, ion-exchanged water, city water, industrial water, an aqueous solution of sodium hydroxide, an aqueous solution of sodium chloride, sea water and mixtures thereof.

6. The method as claimed in claim 1 wherein not less than 0.1 part by volume of said water is added in said step (c-2) to 1 part by volume of said nonpolar solvent phase.

7. The method as claimed in claim 1 wherein said nonpolar solvent in said step (c-3) is selected from hexane, heptane, octane, cyclohexane, benzene, toluene, xylene, petroleum ether, naphtha, kerosene, n-paraffin and mixtures thereof.

8. The method as claimed in claim 1 wherein not less than 0.1 part by volume of said nonpolar solvent is added in said step (c-3) to 1 part by volume of said nonpolar solvent phase.

9. The method as claimed in claim 1 wherein said xanthophyll compound is selected from astaxanthin, canthaxanthin, zeaxanthin, adonixanthin, adonirubin, β-cryptoxanthin, echinenone, asteroidenone, 3-hydroxyechinenone, rhodoxanthin, fucoxanthin, lutein, capsanthin, capsorubin and mixtures thereof.

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(71) Applicant: **NIPPON OIL CO. LTD.**
Minato-ku Tokyo (JP)

(72) Inventors:
- **Kitaoka, Mitomitsu**
  Yokohama, Kanagawa (JP)
- **Tsubokura, Akira**
  Yokohama, Kanagawa (JP)
- **Kiyota, Takashi**
  Yokohama, Kanagawa (JP)

(74) Representative: **Hallybone, Huw George et al**
CARPMAELS AND RANSFORD
43 Bloomsbury Square
London WC1A 2RA (GB)

(54) **Method for purifying xanthophyll compound**

(57) A method for purifying a xanthophyll compound involves the steps of: (a) adding a nonpolar solvent and water to an aqueous organic solvent solution containing the xanthophyll compound; (b) subjecting to liquid-liquid extraction to thereby obtain a nonpolar solvent phase; and (c) subjecting the nonpolar solvent phase to at least one step selected from the following steps (c-1) to (c-3): step (c-1) of concentrating the nonpolar solvent phase; step (c-2) of adding water to the nonpolar solvent phase followed by further subjecting at least once to liquid-liquid extraction; and step (c-3) of further adding a nonpolar solvent to the nonpolar solvent phase whereby the xanthophyll compound is precipitated.

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 30 1665

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	WO 92 01754 A (UNILEVER PLC) * page 7, line 4 - page 8, line 6 * -----	1,2,9	C09B61/00 C09B67/54 C07B63/00 C07C403/00

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C09B
C07B

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12 February 1997	Ketterer, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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(71)(73) Soker/Patenthaver JAN RAA,
Bamsestien 7, 9000 Tromsø,
FRANK HANSEN,
B 602, Stud.heimen Breivang,
Boks 208, 9000 Tromsø.

(72) Oppfinner Søkerne.

(74) Fullmektig Siv.ing. Rolf Dietrichson,
Onsagers Patentkontor, Oslo.

(30) Prioritet begjært Ingen.

(54) Oppfinnelsens benevnelse FREMGANGSMÅTE TIL FREMSTILLING AV ASTAXANTHIN I EN FORM SOM ER EGNET FOR INNLEMMELSE I FOR FOR OPPDRETTFISK.

(57) Sammendrag Fremgangsmåte for ekstraksjon av fargestoffet astaxanthin, som skal anvendes som fargekilde ved oppdrett av laksefisk, fra reke-avfall eller krill, hvorved slikt råstoff i fersk eller syre-konservert form blandes en eller flere ganger med vegetabilsk eller marin triglyceridolje med temperatur på 100 - 250 °C, fortrinnsvis 150 °C, og deretter presses for å oppnå en olje som er anriket med astaxanthin.

(56) Anforte publikasjoner Ingen.

Den foreliggende oppfinnelse angår en fremgangsmåte til fremstilling av astaxanthin fra astaxanthinholdig materiale fra maritime krepsdyr, f.eks. rekeavfall eller krill, i en form som er egnet for innlemmelse i fôr for oppdrettfisk.

Biproduktet etter rensing av reker er hoder og skall. Denne blanding utgjør årlig ca. 16.000 tonn i Norge. Mesteparten behandles som avfall som fabrikkene betaler for å kunne dumpe på fylling. Noe rekeavfall brukes derimot som tilskudd til fiskefôr, særlig i sluttfôringsfasen, for å gi laksefisk den ønskede rødfarge i kjøttet. Rødfargen skyldes karotenoidet astaxanthin som laksefisker kan oppta fra rekeskallet og avleire i muskelen. Det er det samme fargestoff som gir viltlevende laksefisk rødfarget muskel.

Rekeavfallet i Norge inneholder så mye astaxanthin at det teoretisk ville kunne dekke behovet for slik farge til alle norske dambruk. Når bare en liten del brukes på denne måten, skyldes det praktiske vanskeligheter som f.eks. at det råtner fort, at det er vanskelig å male i så små biter at det kan blandes inn i våtfôr uten at våtpellets faller fra hverandre, og at det er voluminøst. Dessuten varierer innholdet av astaxanthin i rekeavfall betydelig fra fabrikk til fabrikk og innen samme fabrikk fra dag til dag. Det er derfor vanskelig å foreskrive en bestemt minimal innblandingsprosess som sikrer god farge. Disse ulempene med bruk av rekeskall som pigment-kilde har åpnet for betydelig salg av et industrielt fremstilt fargestoff, cantaxanthin, til norske dambruk. Dette kan kjøpes som tørt pulver som kan blandes i fôr i foreskrevet mengde. Astaxanthin er imidlertid å foretrekke da det er laksens naturlige pigment og gir mer stabil farge enn cantaxanthin. Det har imidlertid hittil ikke vært mulig økonomisk å fremstille astaxanthin i en form som er egnet for innlemmelse i våtfôr, slik det er nevnt ovenfor.

Man ville kunne tenke seg å ekstrahere astaxanthinet fra råstoffet, idet astaxanthin er oppløselig i oppløsningsmidler som f.eks. aceton, ethanol, eter, heksan og bensin.

Men hvis astaxanthinet senere skal brukes i fôr eller matvarer, må oppløsningsmidlene dampes bort. Dette er kostbart.

Hensikten med den foreliggende oppfinnelse er derfor å skaffe en økonomisk og enkel fremgangsmåte til fremstilling av astaxanthin fra astaxanthinholdig råstoff, f.eks. rekeavfall eller krill, i en form som er egnet for innlemmelse i fôr for oppdrettfisk.

Fremgangsmåten ifølge oppfinnelsen går ut på at råstoffet blandes en eller flere ganger med vegetabilsk eller marin triglyceridolje for ekstraksjon av astaxanthinet, hvoretter råstoffet presses for utvinning av den astaxanthin-anrikede olje. Denne olje kan direkte tilsettes fiskefôr i en mengde som kan justeres i forhold til astaxanthininnholdet i oljen.

Da en triglyceridolje er forholdsvis viskøs, vil den ikke trenge inn i rekeskallet i ønsket grad for effektiv ekstraksjon. Dessuten er fargestoffet i rekeskall oppløst i fettdråper som er omgitt av vannkapper. Slike vannkapper vil avstøte oljen og derfor være en barriere som vil hindre oljen i å komme i kontakt med de fettdråper i rekeskallet som inneholder astaxanthin. Ifølge en videreutvikling av den foreliggende oppfinnelse blir derfor råstoffet før ekstraksjonen forbehandlet med et middels polart oppløsningsmiddel, f.eks. propionsyre, for sprengning av vannkappen rundt de fettdråper astaxanthinet er oppløst i.

Ved lav temperatur er ekstraksjonsgraden lav. Det foretrekkes derfor å utføre ekstraksjonen ved temperaturer på 100-250°C, fortrinnsvis ved ca. 150°C. Eventuelt kan forbehandlingen og ekstraksjonen finne sted samtidig i olje med temperatur over 100°C.

Der er utført forsøk med oppvarming av soyaolje inneholdende 128 mg astaxanthin pr. liter. Oljen ble oppvarmet til forskjellige temperaturer, og det gjenværende astaxanthininnhold ble målt spektrofotometrisk etter avkjøling til værelsestemperatur og dessuten etter en ny oppvarming og avkjøling. Resultatet er vist i tabell 1.

Tabell 1

Gjenværende astaxanthininnhold i ml/g i en olje som opprinnelig inneholdt 128 ml/g

Oppvarming til (°C):	120	130	140	150	160	170	180	190	200
En oppvarming	127	125	125	124	124	123	100	92	88
To oppvarminger	123	123	115	114	118	100	90	82	67

Som det vil ses av tabell 1, må astaxanthin anses å være stabilt i olje ved temperaturer på opptil ca. 170°C.

Råstoffet kan være avfall (hoder og skall) fra maskinell eller manuell rensing av reker, eller det kan være krill. Dette avfall kan først presses i en konvensjonell fiskemelpresse for fjerning av vann og økning av konsentrasjonen av tørrstoff og farge i avfallet. Da rekeskall er lett bedervelig, kan massen konserveres ved iblanding av uorganisk syre eller organisk syre (f.eks. propionsyre, maursyre eller eddiksyre) til pH-verdien blir lavere enn 5. Syrene bidrar til konservering, men fører også til kjemisk oppløsning av kalken i skallet uten at fargestoffet kommer fri. Dette kommer til uttrykk ved at konsentrasjonen av astaxanthin pr. vektenhet presskake av rekeavfall øker, se tabell 2.

Tabell 2 viser forøvrig at astaxanthin er stabilt i surt rekeavfall. En organisk syre bidrar dessuten til at astaxanthinet ekstraheres lettere fra rekeskallet fordi den som middels polart stoff har en viss overflateaktiv virkning. Ferskt eller med syre blandet rekeskall kan deretter blandes med varm vegetabilsk eller marin triglyceridolje, som etter en egnet blandingstid presses ut igjen som astaxanthinanriket olje.

Når olje med en temperatur på f.eks. 150°C tilføres rekeskall, vil vannet i skallet sprutkoke og fettdråpene med astaxanthin derved komme i direkte kontakt og blande seg med den tilsatte olje. Etter en slik behandling av rekeavfallet med varm olje presses blandingen i en egnet presse, slik at oljen med farge kommer fri. For oppnåelse av en effektiv ekstraksjon kan den utpressede olje (etter ny oppvarming)

Tabell 2

Astaxanthininnhold i presskake av ensilert rekeskall, lagret i opptil 21 d ved 4-5°C, og fordeling av diester, monoester og fritt astaxanthin.

Lagringstid (d)	mg astaxanthin pr. kg våtvekt	Herav som diester %	astaxanthin-monoester %	fritt astaxanthin %
0	175	69,5	22,5	8
2	271	-	-	-
6	287	-	-	-
21	274	64	28	8

blandes med presskaken og blandingen presses på ny. Da astaxanthin er stabilt i varm olje, kan den samme olje brukes til å ekstrahere flere porsjoner rekeskall, slik at konsentrasjonen av farge i olje blir svært høy. For produksjon av olje med standardisert konsentrasjon av astaxanthin kan forskjellige oljeekstrakter blandes og eventuelt fortynnes.

Da den olje som brukes ved ekstraksjonen, i seg selv utgjør et næringsmiddel, kan den astaxanthinanrikede olje anvendes direkte for innblanding i fôr for oppdrettfisk.

Eksempel

100 kg rekeavfall som eventuelt kan ha fått tilsatt 10 liter saltsyre og 1,5 liter propionsyre, blandes med 200 l soyaolje (en triglycerid-olje) med en temperatur på 150°C og presses i en fiskemelpresse etter 10 minutters blanding. Konsentrasjonen av astaxanthin i den utpressede olje vil være avhengig av konsentrasjonen i rekeskallet. Ved bruk av ensilert rekeskall har man oppnådd en konsentrasjon på 445 mg astaxanthin pr. liter olje, og med ferskt rekeskall en konsentrasjon på 339 mg astaxanthin pr. liter olje.

P a t e n t k r a v :

1. Fremgangsmåte til fremstilling av astaxanthin fra astaxanthinholdig materiale fra maritime krepsdyr, f.eks. rekeavfall eller krill, i en form som er egnet for innlemmelse i fôr for oppdrettfisk, k a r a k t e r i s e r t v e d at råstoffet blandes en eller flere ganger med vegetabilsk eller marin triglyceridolje for ekstraksjon av astaxanthinet, hvoretter råstoffet presses for utvinning av den astaxanthin-anrikede olje.

2. Fremgangsmåte som angitt i krav 1, k a r a k t e r i s e r t v e d at råstoffet forbehandles med et middels polart oppløsningsmiddel, f.eks. propionsyre, for sprengning av vannkappen rundt de fettdråper astaxanthinet er oppløst i.

3. Fremgangsmåte som angitt i krav 1 eller 2, k a r a k t e r i s e r t v e d at ekstraksjonen foregår ved temperaturer på over 100°C, fortrinnsvis ved ca. 150°C.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT
United States Patent and Trademark Office
Washington, D.C. 20231
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U.S. APPLICATION NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
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GREGORY A NELSON
AKERMAN SENTERFITT & EIDSON
POST OFFICE BOX 3188
WEST PALM BEACH FL 33402-3188

INTERNATIONAL APPLICATION NO.	
PCT/US98/00987	

I.A. FILING DATE	PRIORITY DATE
10/21/98	10/21/98

DATE MAILED: 08/13/01

NOTIFICATION OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

1. The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as ☐ a Designated Office (37 CFR 1.494), ☒ an Elected Office (37 CFR 1.495), has determined that the above-identified international application has met the requirements of 35 U.S.C. 371, and is **ACCEPTED** for national patentability examination in the United States Patent and Trademark Office.

2. The United States Application Number assigned to the application is shown above and the relevant dates are:

25 Jul 01	25 Jul 01
DATE OF RECEIPT OF 35 U.S.C. 371(c)(1), (c)(2) and (c)(4) REQUIREMENTS	DATE OF RECEIPT OF ALL 35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** *The filing date of the above-identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363).* Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

3. ☒ A request for immediate examination under 35 U.S.C. 371(f) was received on 30 Apr 01 and the application will be examined in turn.

4. The following items have been received:
- ☒ U.S. Basic National Fee.
- ☒ Copy of the international application.
- ☐ Translation of the international application into English.
- ☒ Oath or Declaration of inventors(s).
- ☐ Copy of Article 19 amendments. ☐ Translation of Article 19 amendments into English.
  The Article 19 amendments ☐ have ☐ not been entered.
- ☐ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Copy of the Annexes to the International Preliminary Examination Report (IPER).
  ☐ Translation of Annexes to the IPER into English.
  The Annexes ☐ have ☐ not been entered.
- ☒ Preliminary amendment(s) filed 30 Apr 01 and _____
- ☐ Information Disclosure Statement(s) filed _____ and _____.
- ☐ Assignment document.
- ☒ Power of Attorney and/or Change of Address.
- ☐ Substitute specification filed _____.
- ☐ Indication of Small Entity Status.
- ☒ Priority Document.
- ☒ Copy of the International Search Report ☑ and copies of the references cited therein.
- ☐ Other:

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5).

Lamont Hunter, Paralegal
Telephone: 703 305-3686

FORM PCT/DO/EO/903 (March 2001)

04-23

JC17 Rec'd PCT/PTO 20 APR 2001

Form PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 789-47
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/830146
INTERNATIONAL APPLICATION NO. PCT/CA99/00987 APR 2 0 2001	INTERNATIONAL FILING DATE 21 October 1999	PRIORITY DATE CLAIMED 21 October 1998
TITLE OF INVENTION METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES		
APPLICANT(S) FOR DO/EO/US BEAUDOIN, Adrien; MARTIN, Geneviève		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) [unsigned].

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [x] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [ ] Other items or information:

EXPRESS MAIL LABEL NO. EL 740156190 US

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/830146	INTERNATIONAL APPLICATION NO. PCT/CA99/00987	ATTORNEY'S DOCKET NUMBER 789-47

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total claims	44	-20 =	14	X **$18.00**	$252.00	
Independent claims	5	-3 =	2	X **$80.00**	$160.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =					$1272.00	
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					$636.00	
SUBTOTAL =					$636.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.429(f)). +					$	
TOTAL NATIONAL FEE =					$636.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					$	
TOTAL FEES ENCLOSED =					$636.00	
					Amount to be: refunded	$
					Charged	$

a. [X] A check in the amount of $ 636.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0951 in the amount of $ 0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0951. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Gregory A. Nelson
Akerman, Senterfitt & Eidson, P.A.
Post Office Box 3188
West Palm Beach, FL 33402-3188

SIGNATURE

Joseph W. Bain
NAME

34,290
REGISTRATION NUMBER

FORM PTO-1390 (REV 11-98) page 2 of 2

P1011358;1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

PRELIMINARY AMENDMENT

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Pages 29 and 30 were unintentionally omitted from the response to the Written Opinion during International Preliminary Examination. Please therefore insert the following text after table 15 and before the claims:

- - TABLE 16. 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 17. 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.

TABLE 18. 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 19: 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 activity	enzymatic

			(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 - -

IN THE CLAIMS:

1. (Amended) 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

(g) recovering the solid contents.

4. (Amended) A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. (Amended) A method as in claim 1, wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. (Amended) A method as in claim 1 , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

7. (Amended) A method as in claim 1, 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).

8. (Amended) A method as in claim 1, 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. (Amended) A method as in claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

10. (Amended) A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

11. (Amended) A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

Please cancel claim 13 without prejudice.

14. (Amended) A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

Please cancel claim 18 without prejudice

19. (Amended) A method as in claim 15, wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in claim 15, wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

25. (Amended) A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

27. (Amended) A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

Please cancel claims 28 and 29 without prejudice

30. (Amended) A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Please cancel claims 34 and 35 without prejudice.

36. (New) A method as in claim 1, wherein the ketone solvent is acetone.

37. (New) A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

38. (New) A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

39. (New) A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

40. (New) A method as in claim 15, wherein said marine and aquatic animal material is viscera.

41. (New) A method as in claim 15, wherein the ketone solvent is acetone.

42. (New) A method as in claim 16, wherein said marine and aquatic animal material is viscera.

43. (New) A method as in claim 16, wherein the animal material is selected from krill and *Calanus.*

44. (New) A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

45. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

46. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

47. (New) A method as in claim 1, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

48. (New) A method as in claim 31, wherein the ketone solvent is acetone.

49. (New) A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

50. (New) A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

51. (New) A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

REMARKS

A complete copy of the specification which is to form the basis for the US National Phase of PCT/CA99/00987 is submitted herewith.

Respectfully submitted

Date: 4/20/01

Gregory A. Nelson
Registration No. 30,577
Joseph W. Bain
Registration No. 34,290
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, Suite 400
P.O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: 561-653-5000

Docket No. 789-47

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

ATTACHMENT TO PRELIMINARY AMENDMENT SHOWING MODIFICATIONS

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

In accordance with 37 CFR §1.121, the modifications made to the specification and claims are as follows:

IN THE SPECIFICATION

After table 15 and before the claims (modifications indicated with respect to the PCT application as originally filed)

TABLE [17]16. 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]17. 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.

TABLE [19]18. 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]19: 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 activity	enzymatic

			(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

IN THE CLAIMS

Modifications indicated with respect to the claims existing after International Preliminary Examination

1. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(n) 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;

(o) separating the liquid and solid contents;

(p) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(q) 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;

(r) separating the liquid and solid contents;

(s) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(t) recovering the solid contents.

4. (Amended) A method as in [any of] claim[s] 1 [to 3], wherein steps (b) and (d) are conducted under inert gas atmosphere.

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

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

7. (Amended) A method as in [any of] claim[s] 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).

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

10. (Amended) A method as in claim[s] 1 [to 9], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

11. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

14. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a 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 an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a 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 solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15 [or 16], wherein the animal material is selected from krill and *Calanus*.

19. (Amended) A method as in [any one of] claim[s] 15 [to18], wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in [any one of] claim[s] 15 [to 19], wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in [any one of] claim[s] 15 [to 20], wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in [any one of] claim[s] 15 [to21], wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in [any one of] claim[s] 15 [to 22], wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein prior to step (a) the marine and aquatic animal material is finely divided[, preferably to an average particle size of 5mm or less].

25. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 [and preferably at least about 90] mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g [and preferably at least about 270 mg/g] of krill extract.

27. (Amended) A method of lipid extraction as in [any one of] claim[s] 1 [to 14], wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

30. (Amended) A method of lipid extraction as in [any one of] claim[s] 15 [to 25], wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) 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 of step (b) 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 of step (e);

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) [The] A lipid fraction [obtained by the method of any one of claims 1 to 25, 27, and 30 to 32] extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Respectfully submitted

Date: 4/20/01

Gregory A. Nelson
Registration No. 30,577
Joseph W. Bain
Registration No. 34,290
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, Suite 400
P.O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: 561-653-5000

Docket No. 789-47

TITLE OF THE INVENTION

METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

FIELD OF THE INVENTION

This invention relates to the extraction of lipid fractions from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals. More specifically, this invention relates to an improved method of extracting lipid fractions by dehydration with solvents and recovering a solid residue rich in active enzymes.

BACKGROUND OF THE INVENTION

Lipid fractions obtained from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals have various applications:

Medical applications

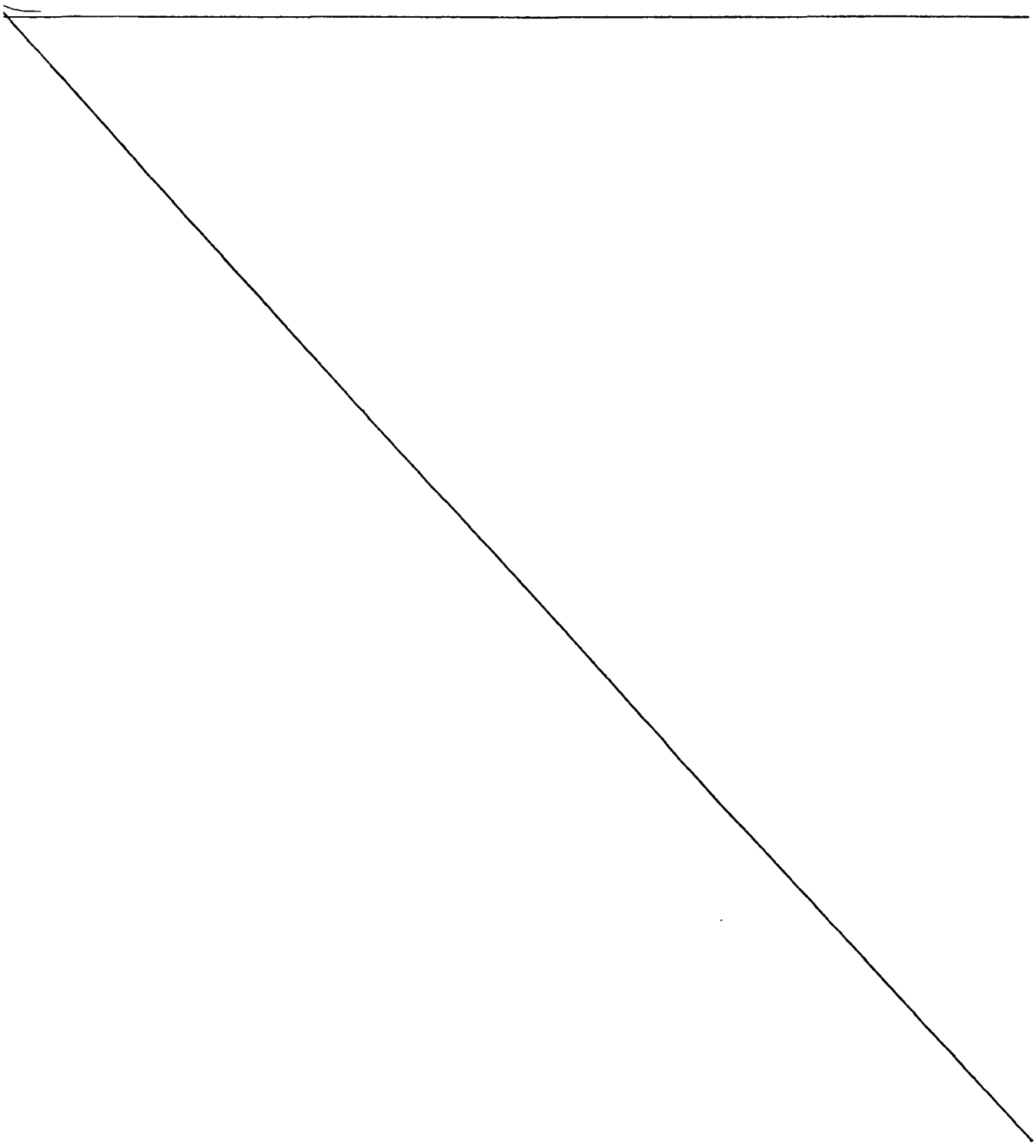
Marine and aquatic animal oils and fractions thereof contain various therapeutic agents. For example, it is reported that various marine and aquatic animal oils have anti-inflammatory properties. Marine and aquatic animal oils are also reported as helpful in reducing the incidence of cardiovascular disease. Also, some marine and aquatic animal oils are reported as suppressing the development of certain forms of lupus and renal diseases. As a further example, krill may be used as a source of enzymes for debridement of ulcers and wounds or to facilitate food digestion. Also marine and aquatic oils contain various antioxidants, which may have potential therapeutic properties.

Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. These fatty acids are essential for proper development of the brain and the eye. Marine and aquatic animal oils are also rich in liposoluble vitamins A, D and E and carotenoids.

Cosmetics

Various marine and aquatic animal oils are used for the production of moisturizing creams.



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 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"* 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 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 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.

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)

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)

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 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 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 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 (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.

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 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 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 reference for measuring efficiency of the extraction process. Another comparison has been made with a technique using hexane as the extraction solvent. Lipid recovery

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 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 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) including some modifications to the original technique: 2h at 65°C 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.

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. The same 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, astaxanthin 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.

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 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).

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

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.

Tables 12 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 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 14 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 12 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 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 12, 13, 14, 15, 16 and 17. It is noteworthy to mention that in Table 17, the carotenoids content was significantly high as measured in terms of two carotenoids namely astaxanthin and canthaxanthin. Indeed, duplicates analyzes revealed values of 92 to 124 µg/g of lipid fraction for astaxanthin 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 astaxanthin at least 75 and preferably at least 90 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 (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.

Table 18 shows the best mode of the method in accordance with the present invention for lipid extraction of aquatic animal tissues.

Table 19 shows that the enzyme activity of the solid fraction is maintained following 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 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].

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.

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 (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)

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

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 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.

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.

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.

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.

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.

BIBLIOGRAPHY

Bowyer, D.E., Leat, W.M.F., Howard, A.N. and Gresham, G.A. 1962. The determination of the fatty acid composition of serum lipids separated by thin-layer chromatography; and a comparison with column chromatography. BBA. 70: 423-431.

Chandrasekar, B., Troyer, D.A., Venkatraman, J.T. and Fernandes, G. 1996. Tissue specific regulation of transforming growth factor beta by omega-3 lipid-rich krill oil in autoimmune murine lupus. Nutr Res. 16(3): 489-503.

Christensen, M.S., Hoy, C-E. and Redgrave, T.G. 1994. Lymphatic absorption of *n*-3 polyunsaturated fatty acids from marine oils with different intramolecular fatty acid distributions. BBA. 1215: 198-204.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. 1983. Spectrophotometric assay using *o*- Phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci. 66: 1219-1227.

Difco laboratories. 1984. Difco Manual Dehydrated Culture Media and Reagents for Microbiology. 10th ed. Detroit.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem. 226: 497-509.

Goodman Gilman, A., Goodman, L.L. and Gilman, A. 1980. The Pharmacological Basis of Therapeutics. 6th ed. Collier Macmillan Canada ltd, Toronto.

Harwood, H.J. and Geyer, R.P. 1964. Biology Data Book. The Federation of American Societies for Experimental Biology, Washington.

Hellgren, L., Karlstam, B., Mohr, V. and Vincent, J. 1991. Krill enzymes. A new concept for efficient debridement of necrotic ulcers. Int J Dermatol. 30(2): 102-103

Plummer, D.T. 1987. An introduction to practical biochemistry. 3th ed. McGraw-Hill Book Company, London.

Rawn, J.D. 1990. Traité de biochimie. De Boeck-Wesmael, Bruxelles.

Runge, J.A. and Joly, P. 1994. Rapport sur l'état des invertébrés en 1994: 7:0 Zooplancton (Euphausiacés et *Calanus*) de l'Estuaire et du Golfe du Saint-Laurent.

Sargent, J.R. 1997. Fish oils and human diet. Br J Nutr.78 Suppl 1: S5-S13.

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

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

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
7-	acetone [a]	2,15		
	t-butanol [c]	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]	2,28		
	ethyl acetate [b]	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**	

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.

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.

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..

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.

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 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**
2-	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. 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 ™.

TABLE 13. 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 14. 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 14 (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 14 (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 15. 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

TABLE 15 (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.

TABLE 16. 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 17. 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.

TABLE 18. 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 19: 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

CLAIMS

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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

(g) recovering the solid contents.

4. A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

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

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

7. A method as in claim 1, 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).

8. A method as in claim 1, 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 claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

10. A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

11. A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

12. A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

14. A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

15. A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16. A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

19. A method as in claim 15, wherein during step (a), the animal material is homogenized.

20. A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. A method as in claim 15, wherein after step (b) and before step (c), the method

additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

25. A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

26. A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

27. A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

30. A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

whereby lipid fractions are obtained.

32. A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

33. A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

36. A method as in claim 1, wherein the ketone solvent is acetone.

37. A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

38. A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

39. A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

40. A method as in claim 15, wherein said marine and aquatic animal material is viscera.

41. A method as in claim 15, wherein the ketone solvent is acetone.

42. A method as in claim 16, wherein said marine and aquatic animal material is viscera.

43. A method as in claim 16, wherein the animal material is selected from krill and *Calanus*.

44. A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

45. A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

46. A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

47. A method as in claim 1, wherein the solid contents of step (e) is recovered and

consists of a dehydrated residue containing active enzymes.

48. A method as in claim 31, wherein the ketone solvent is acetone.

49. A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

50. A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

51. A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

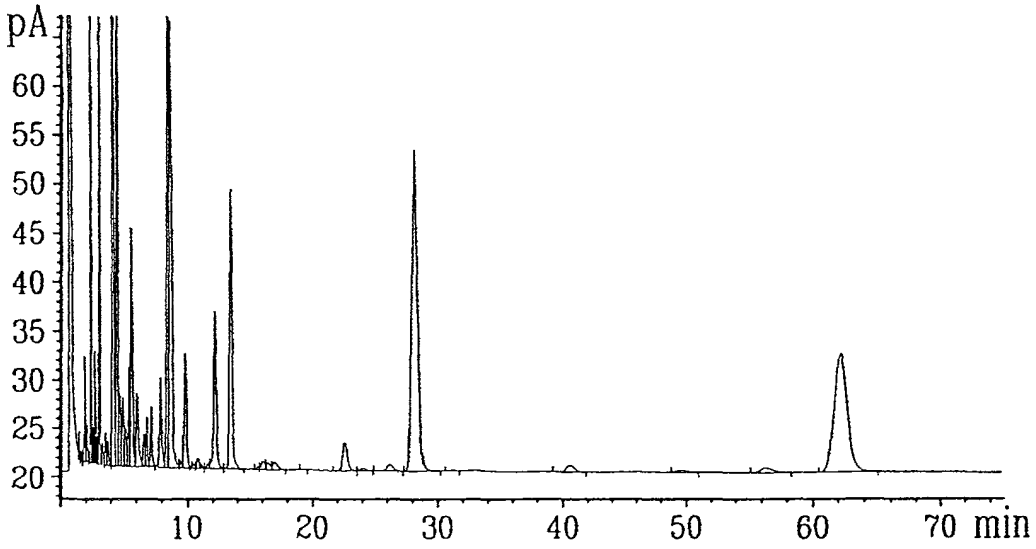


Fig. 1

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	

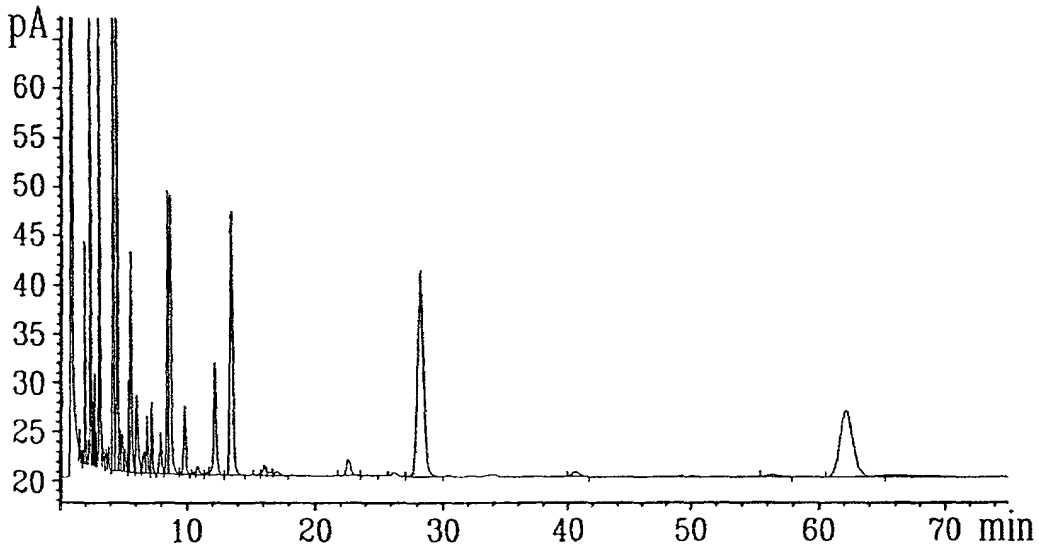


FIG. 2

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

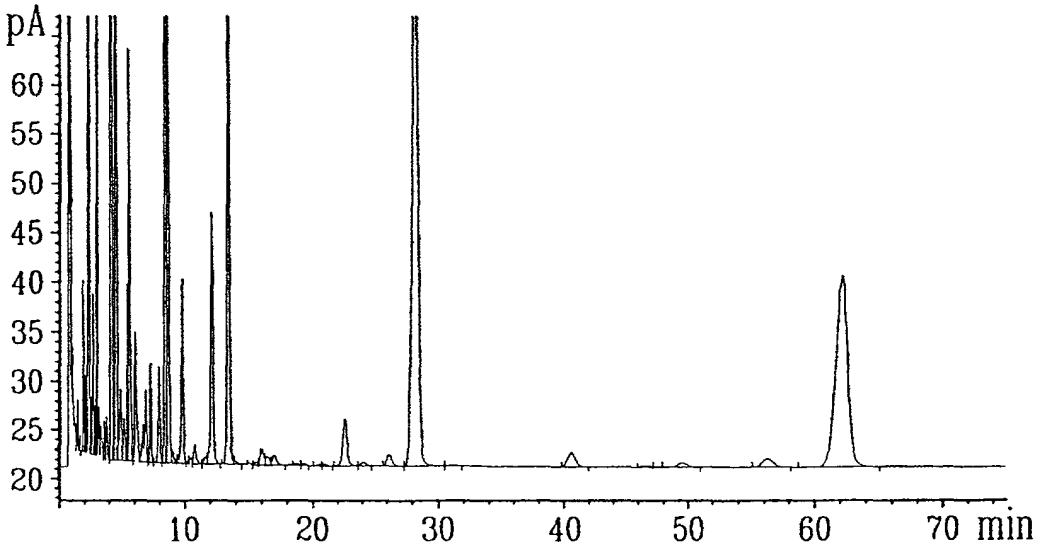


Fig 3

1.216	4.520 - 16:1	13.457
1.262	4.683	13.943
1.454 - 12:0	4.884 - 16:1tr	15.053
1.624	5.030	15.572 - 20:0
1.811	5.111	16.016
1.875	5.420	16.486 - 20:1
2.016	5.561	16.999 - 20:1(cis11)
2.054	6.031	18.762
2.174	6.642	19.303 - 20:2
2.330 - 14:0	6.868	20.474
2.505	7.226	21.027 - 20:3
2.589 - 14:1	7.908 - 18:0	22.575 - 20:4 (6,10,14,18)
2.679	8.444 - 18:1	24.071
2.799	8.639 - 18:1tr	26.215
2.854	9.005	28.333
2.981	9.536	31.180
3.074 - std 15:0	9.788 - 18:2	40.560
3.304	10.267	46.595
3.580	10.481	49.513
3.804	10.807	56.292
4.169 - 16:0	11.626	62.250
4.296	12.140 - 18:3	

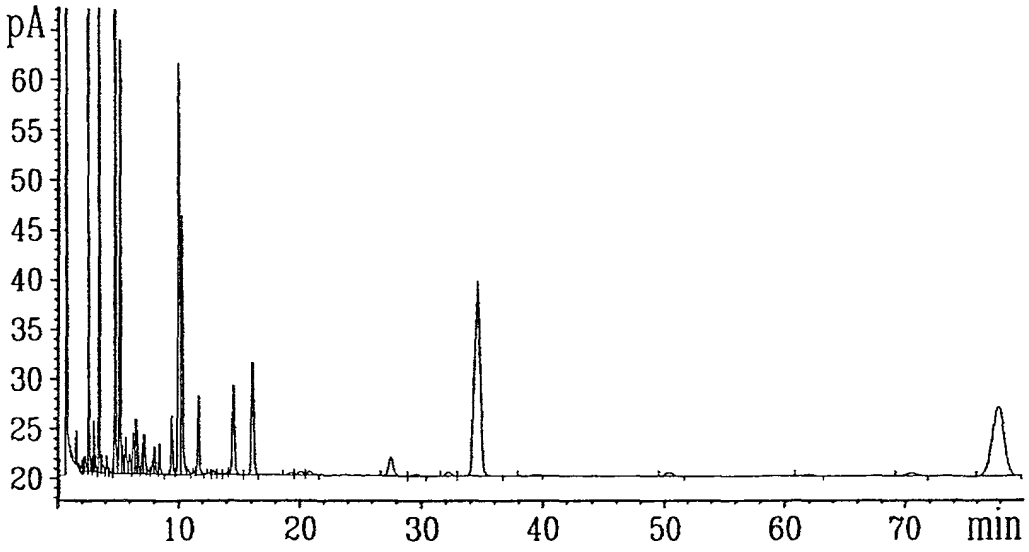


Fig 4

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	

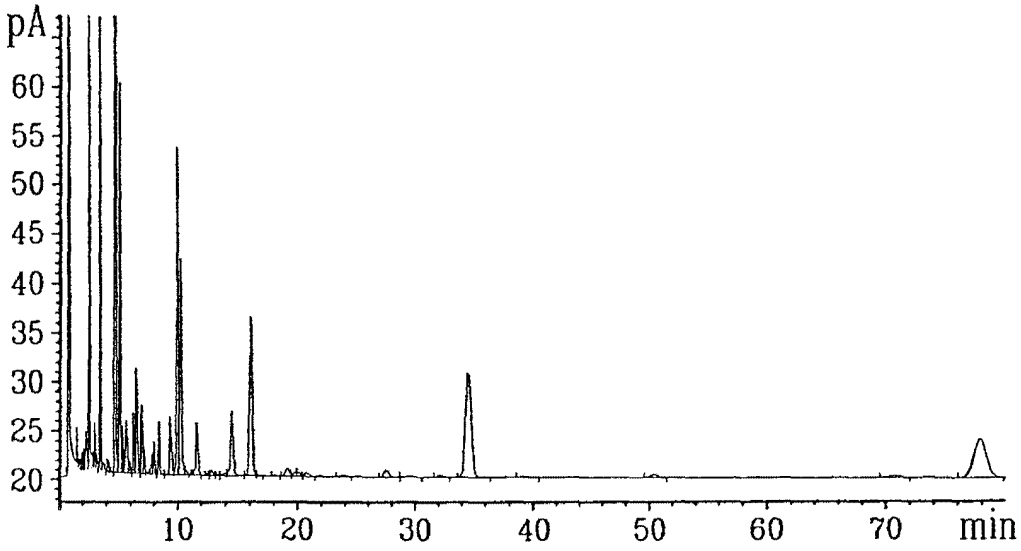


Fig 5

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	

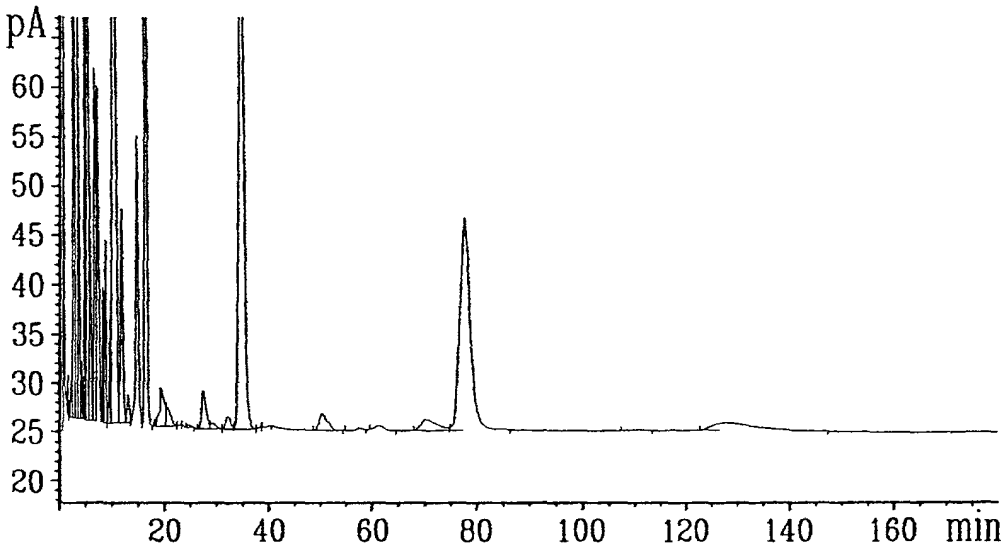


Fig 6

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	

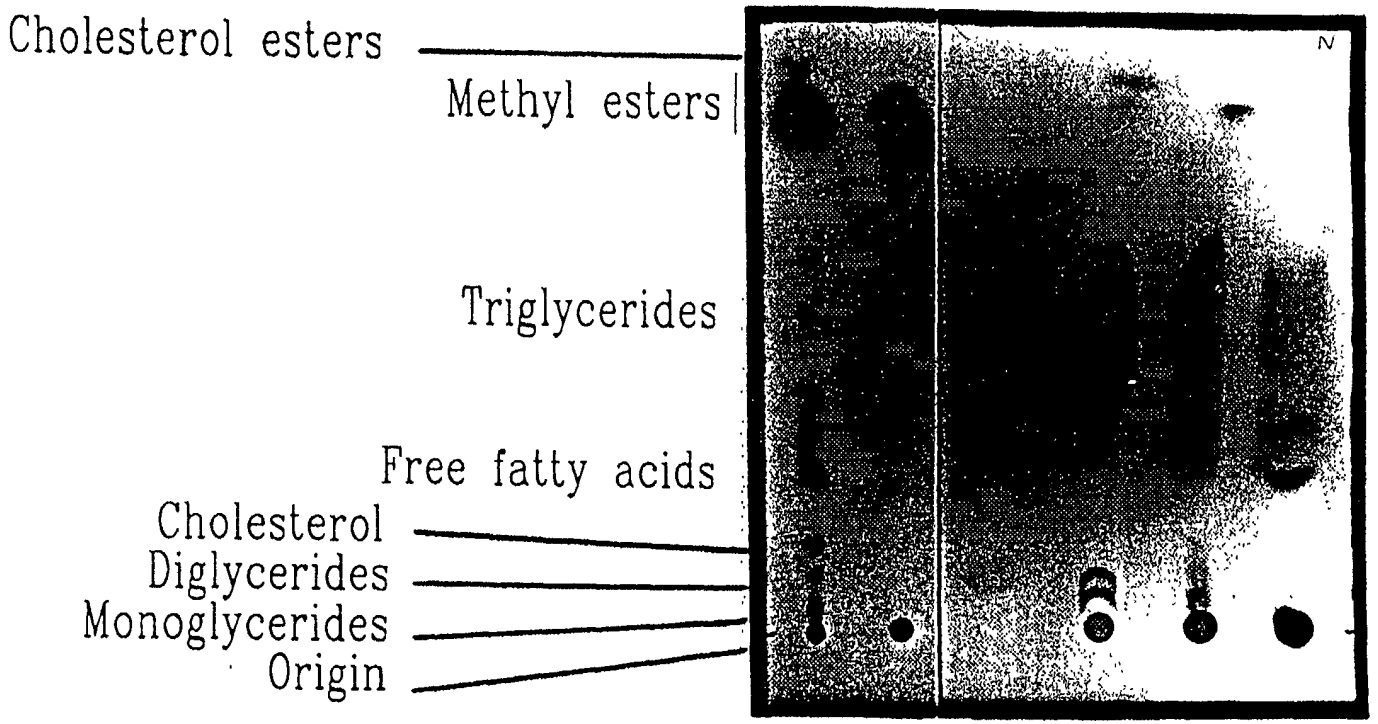


FIG. 7

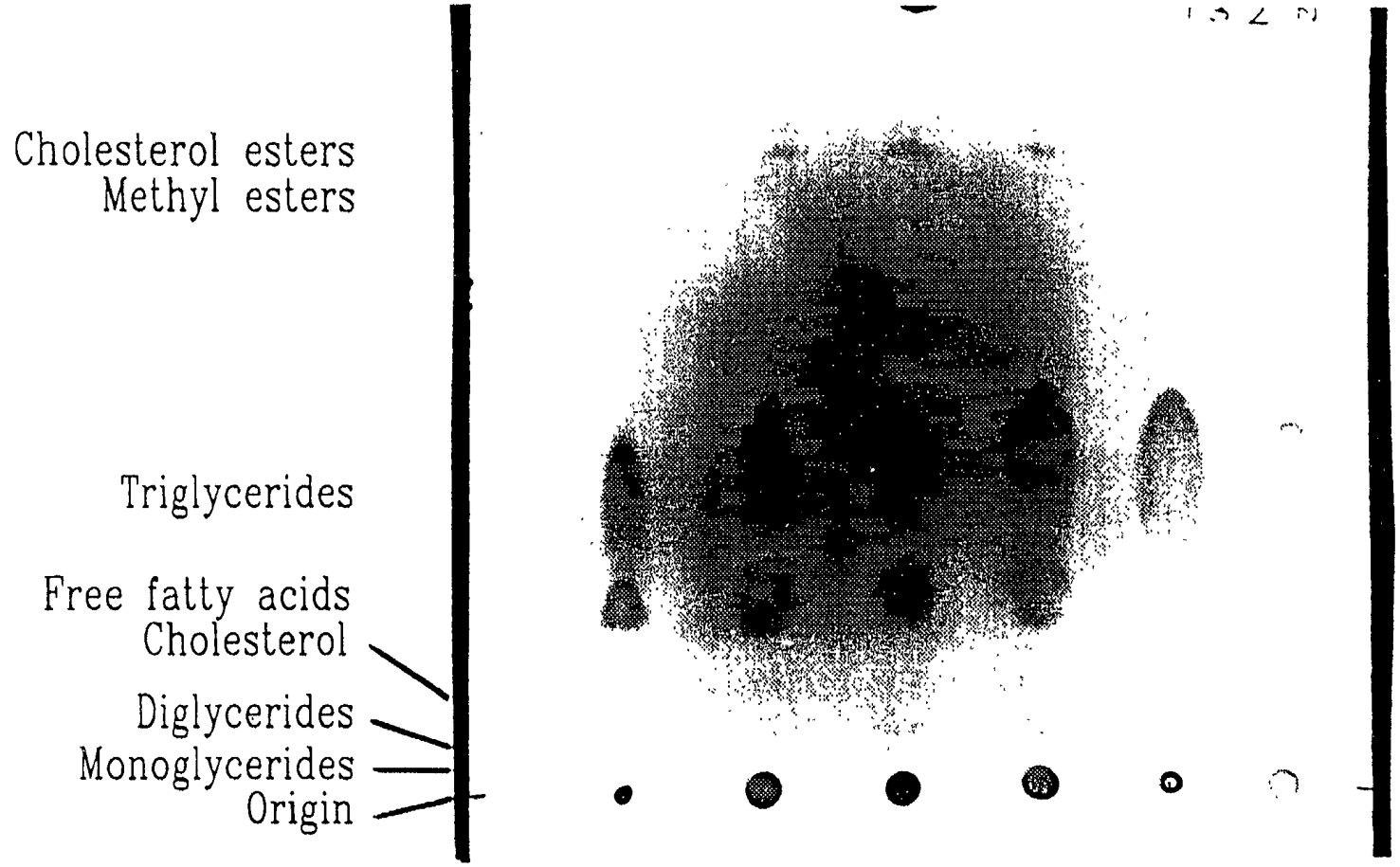


FIG. 8

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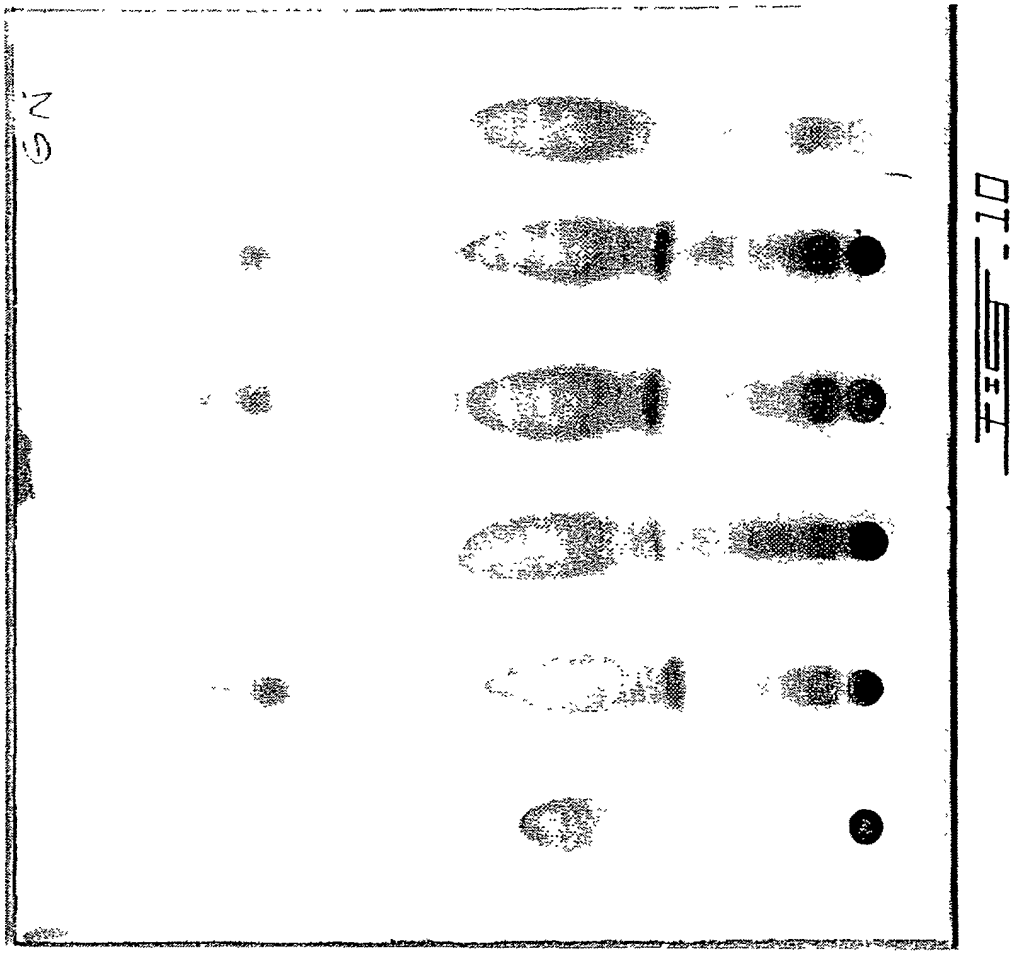
Fig. 5



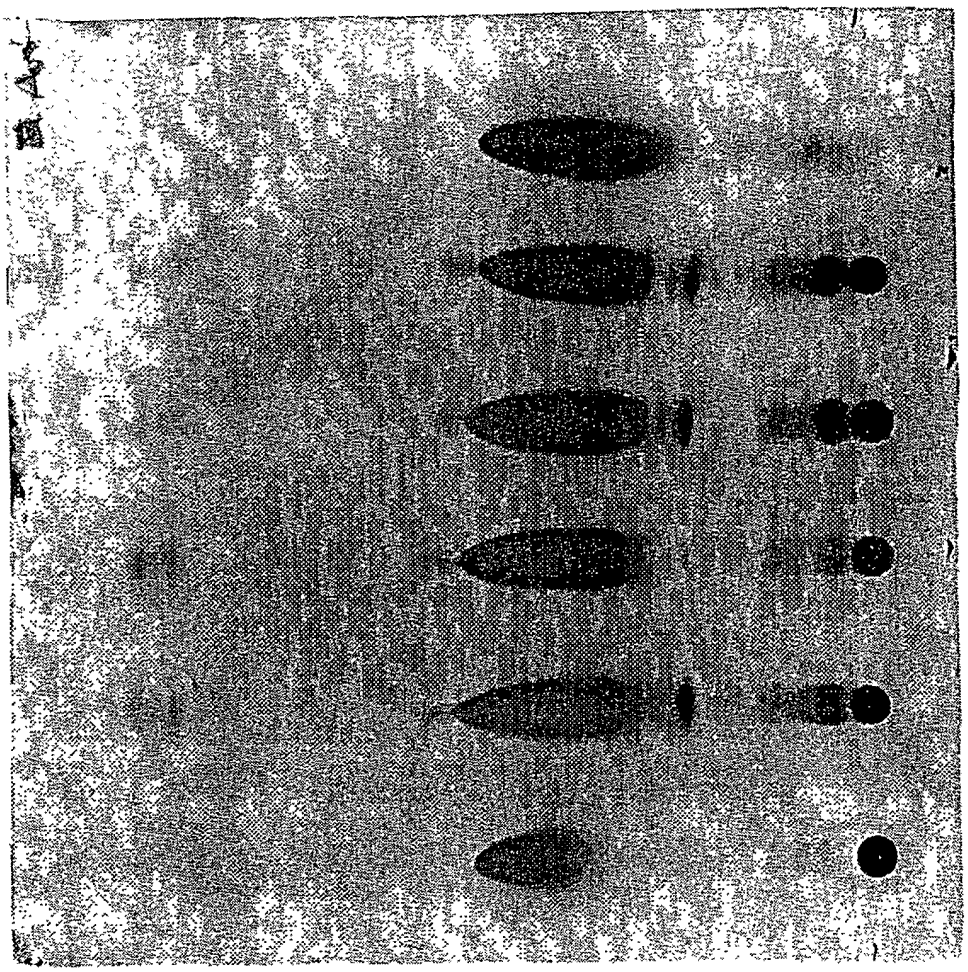


Fig. 11

A B C D E F G H

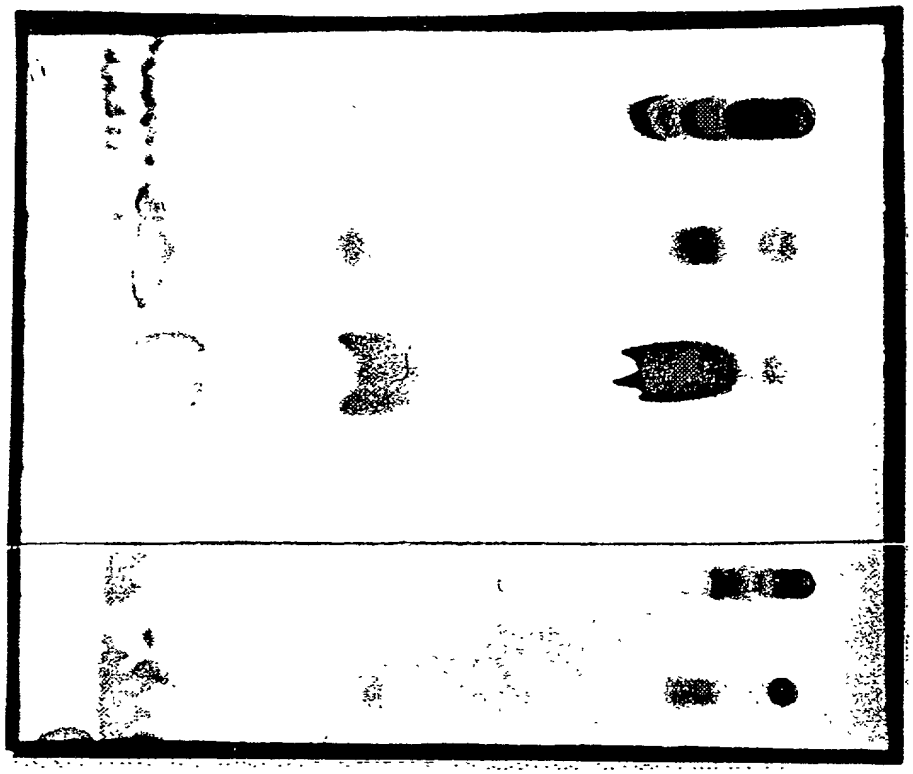


Fig. 12

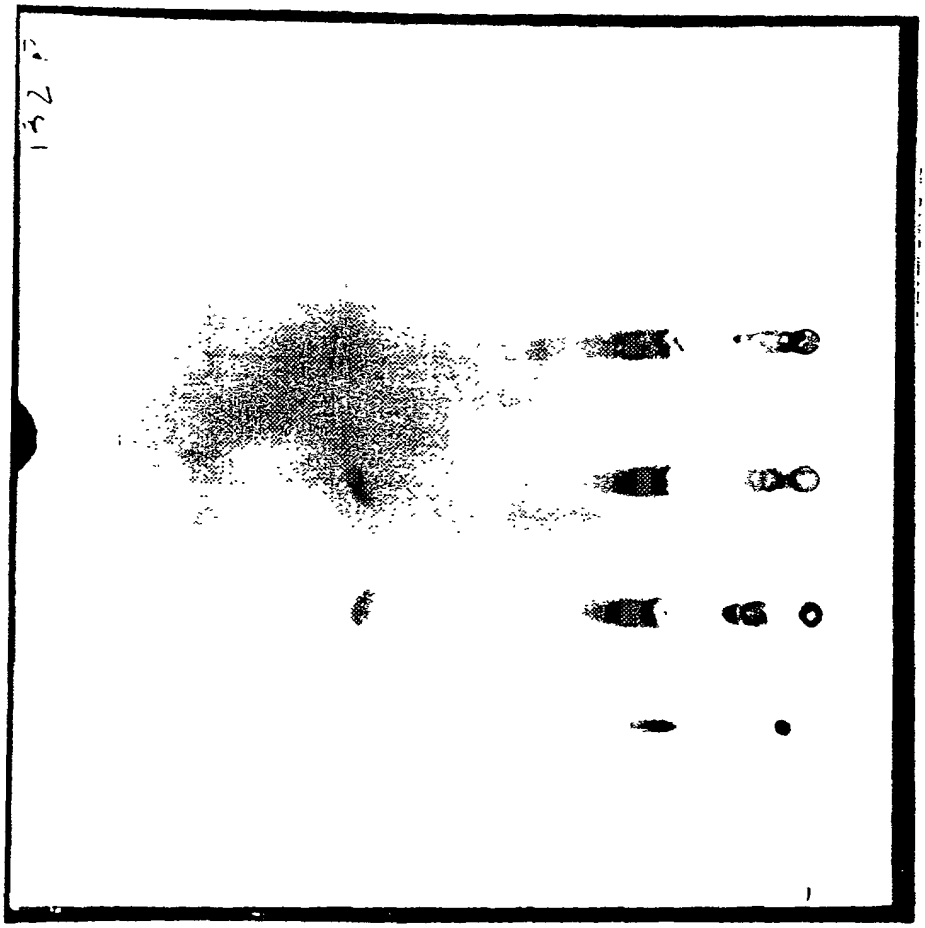


Fig. 13

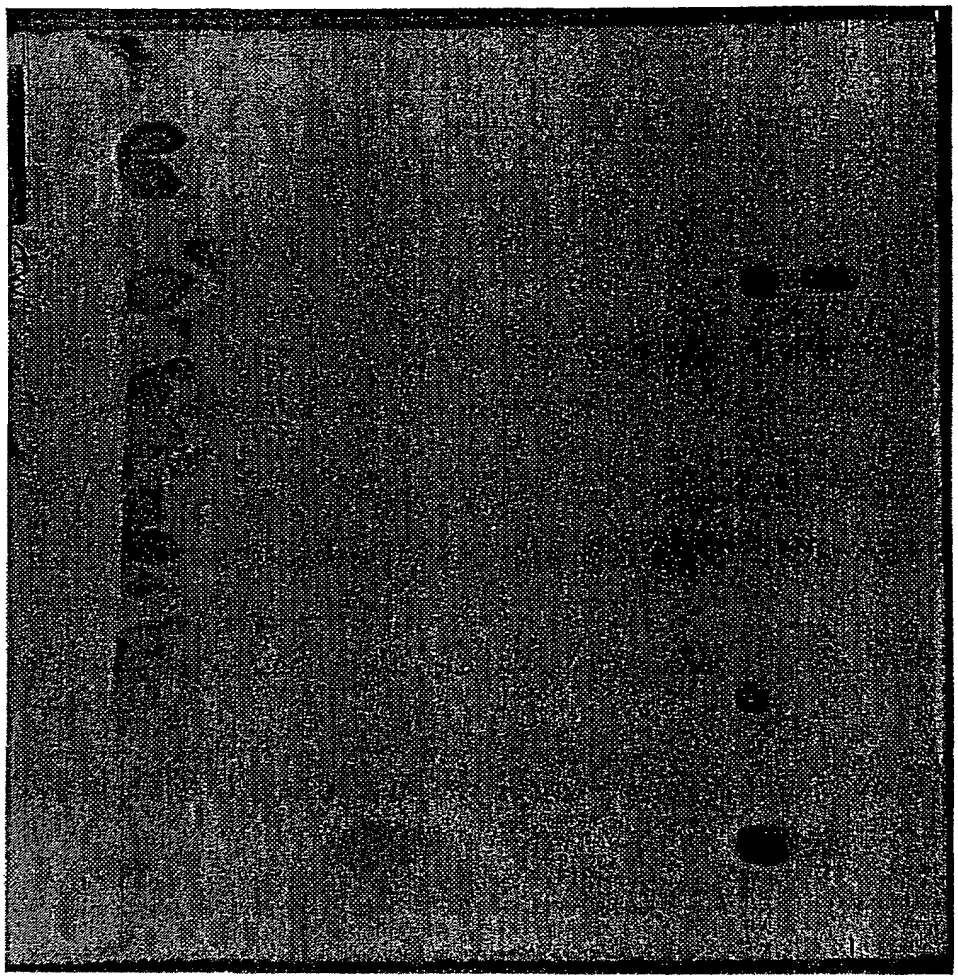


Fig-14

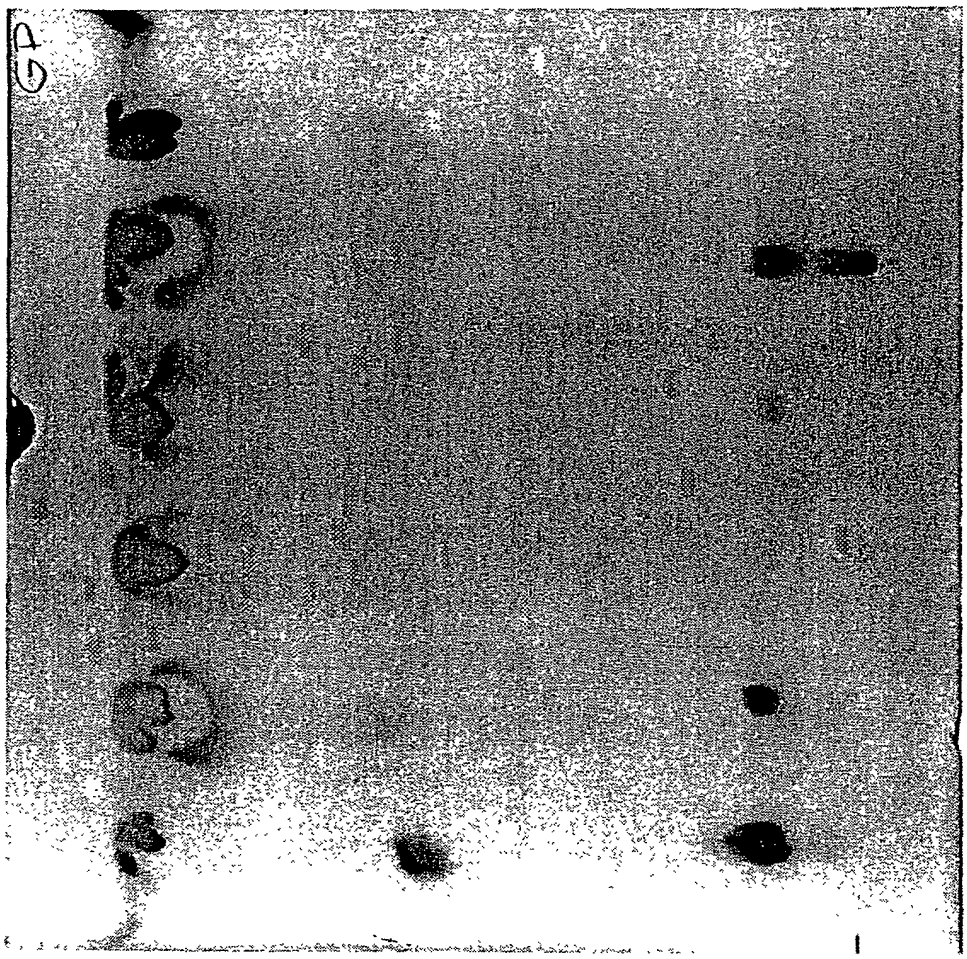


Fig-15

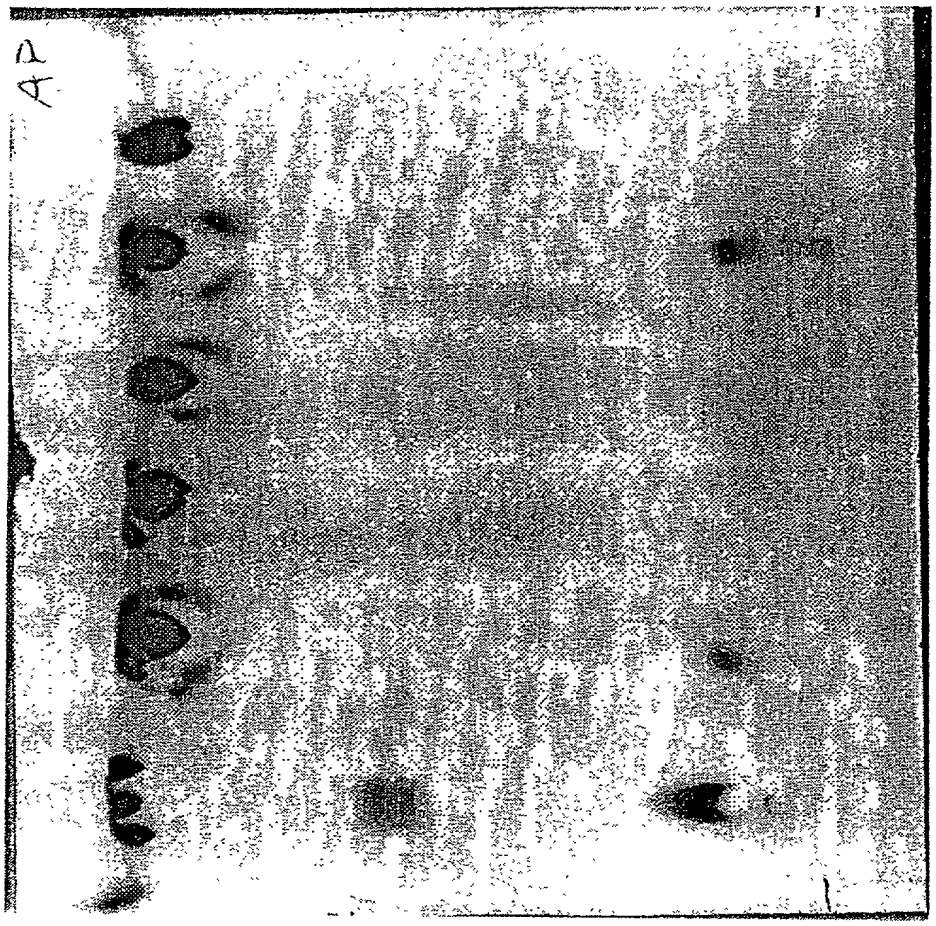


Fig-16

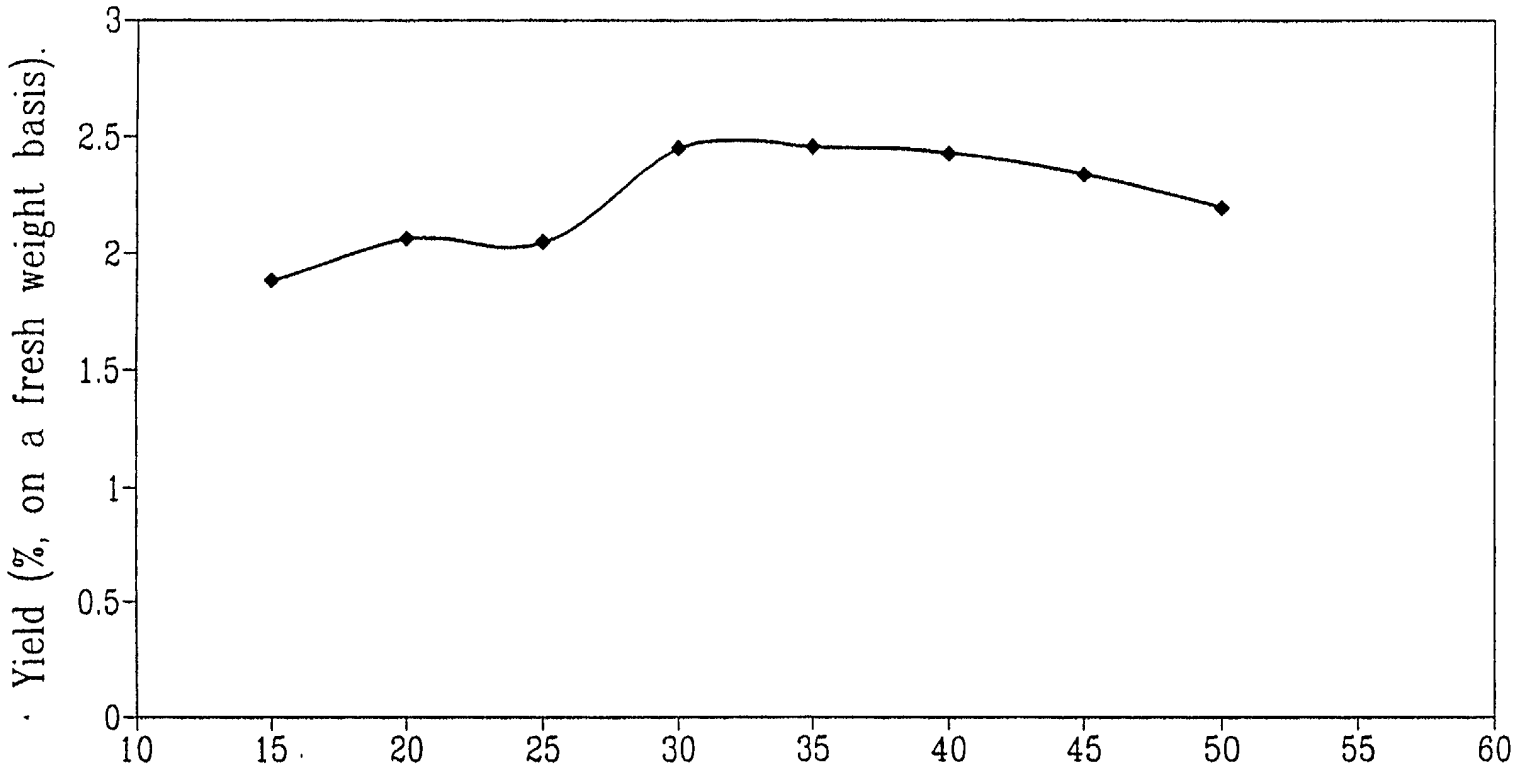


Fig. 17

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09/830146

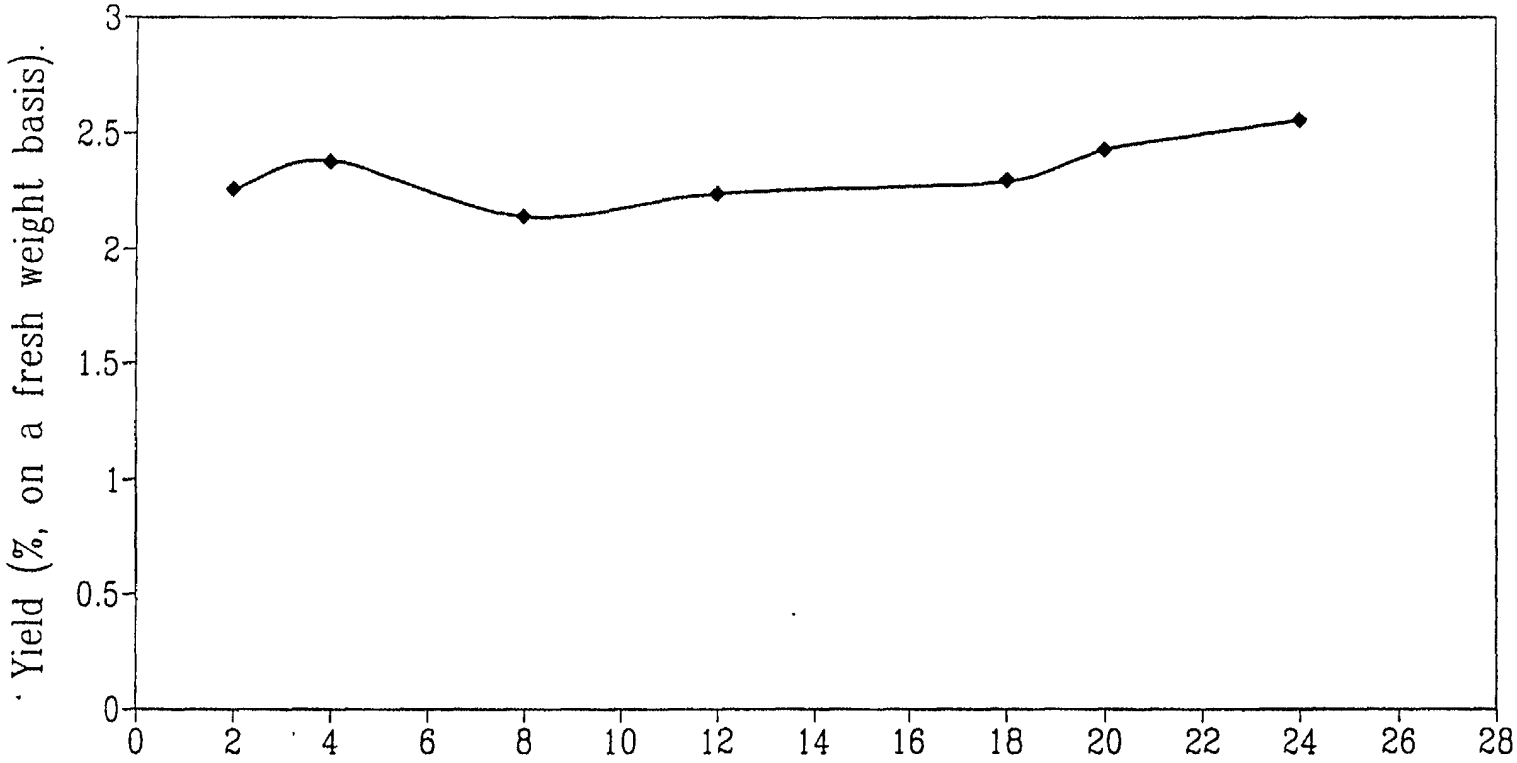

Fig. 18

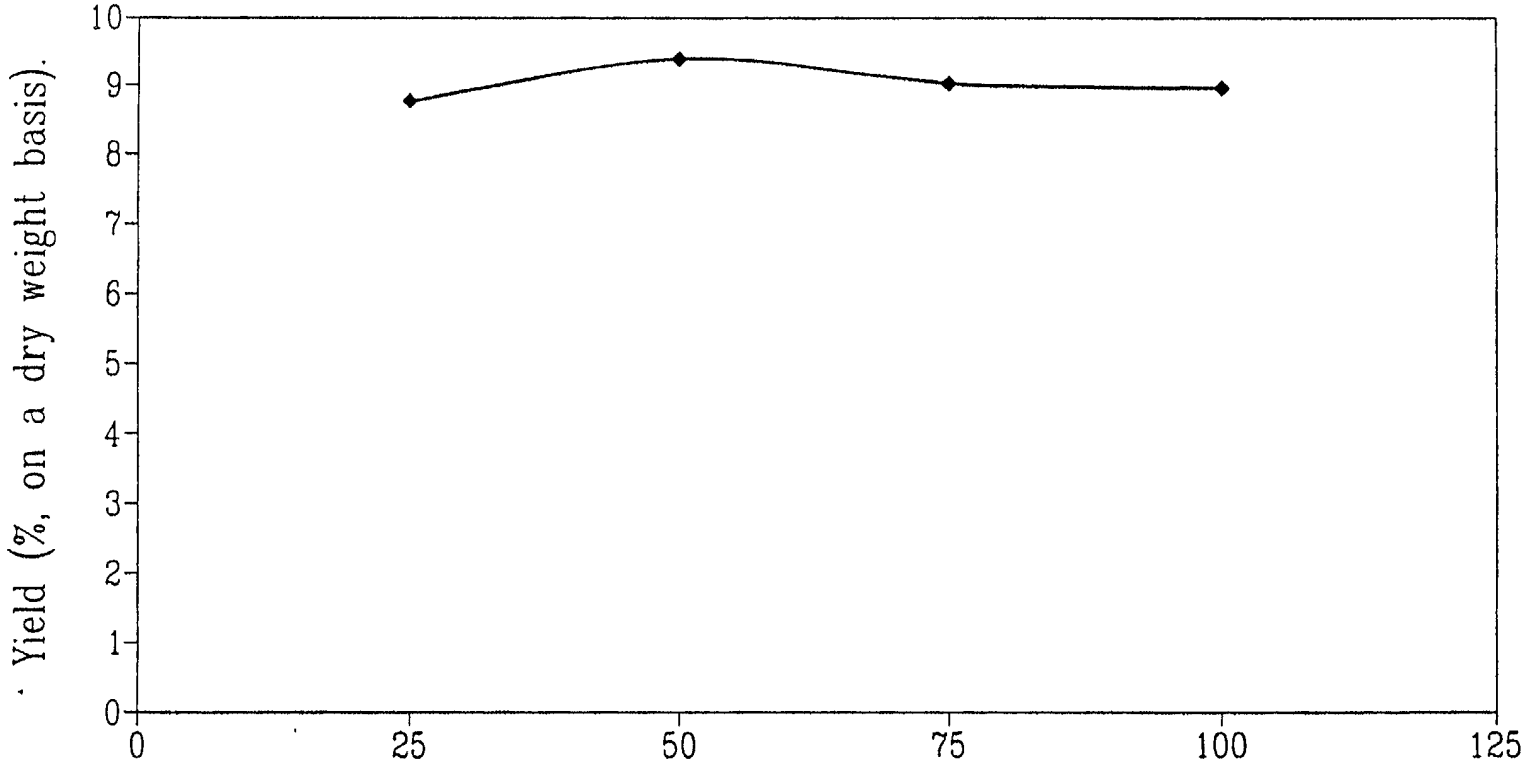

Fig. 19

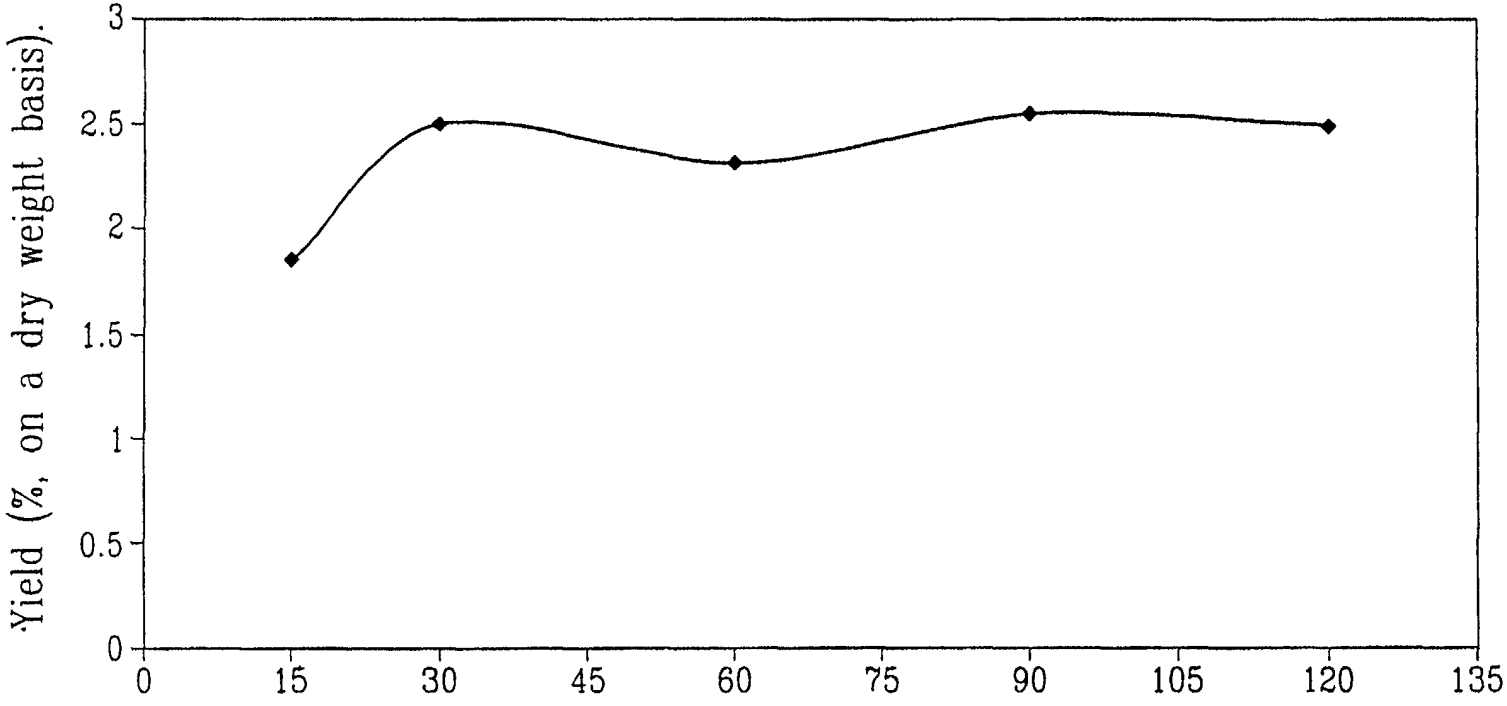

FIG. 20

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	ATTORNEY DOCKET NUMBER 789-47

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

---

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _
on _,
as amended on _ (if applicable).

[x] was filed as a PCT international application number PCT/CA99/00987 on 21 October 1999 as amended under PCT Article 19 on __ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
CANADA	2,251,265	21 October 1998	[x] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U S FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS			
PCT/CA99/00987	21 October 1999				

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith: J. Rodman Steele, Jr., Registration No. 25,931; Gregory A. Nelson, Registration No. 30,577, Joseph W. Bain, Registration No. 34,290; Robert J. Sacco, Registration No. 35,667; Mark D. Passler, Registration No. 40,764; Stanley A. Kim, Registration No. 42,730; Steven M. Greenberg, Registration No. 44,725; Neil R. Jetter, Registration No. 46,803; Larry G. Brown, Registration No. 45,834; Kevin T. Cuenot, Registration No. 46,283; Pablo Meles, Registration No. 33,739; Raynaldo K. Whitty, Registration No. 47,176; and Barbara S. Kitchell, Registration No. 33,928.

Send Correspondence to:
Akerman, Senterfitt & Eidson, P.A.
Post Office Box 3188
West Palm Beach, FL 33402-3188

Direct Telephone Calls to:
(561) 653-5000

	FULL NAME OF INVENTOR	FAMILY NAME: BEAUDOIN	FIRST GIVEN NAME: Adrien	SECOND GIVEN NAME
201	RESIDENCE & CITIZENSHIP	CITY: Rock Forest CAX	STATE OR COUNTRY: Quebec, Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 748, boulevard des Vétérans	CITY: Rock Forest	STATE & ZIP CODE/COUNTRY: Quebec, J1N 1Z7, Canada
	FULL NAME OF INVENTOR	FAMILY NAME: MARTIN	FIRST GIVEN NAME: Geneviève	SECOND GIVEN NAME
202	RESIDENCE & CITIZENSHIP	CITY: Sherbrooke	STATE OR COUNTRY: Quebec. Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 979, McManamy	CITY: Sherbrooke	STATE & ZIP CODE/COUNTRY: Quebec, J1H 2N1, Canada
	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
203	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
A Beaudoin		
DATE: 31 juillet 2001	DATE	DATE

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	ATTORNEY DOCKET NUMBER 789-47

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My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

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the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _ on _, as amended on _ (if applicable).

[x] was filed as a PCT international application number PCT/CA99/00987 on 21 October 1999 as amended under PCT Article 19 on __ (if applicable).

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			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

PTC 1391 Rev 10-83 Page 1 of 2 US DEPARTMENT OF COMMERCE Patent and Trademark Office

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(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER 789-47

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U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U S. SERIAL NUMBERS			
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Direct Telephone Calls to:
(561) 653-5000

	FULL NAME OF INVENTOR	FAMILY NAME BEAUDOIN	FIRST GIVEN NAME Adrien	SECOND GIVEN NAME
201	RESIDENCE & CITIZENSHIP	CITY Rock Forest	STATE OR COUNTRY Quebec, Canada	COUNTRY OF CITIZENSHIP Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 748, boulevard des Vétérans	CITY Rock Forest	STATE & ZIP CODE/COUNTRY Quebec, J1N 1Z7, Canada
	FULL NAME OF INVENTOR	FAMILY NAME MARTIN	FIRST GIVEN NAME Geneviève	SECOND GIVEN NAME
202	RESIDENCE & CITIZENSHIP	CITY Sherbrooke CAX	STATE OR COUNTRY Quebec. Canada	COUNTRY OF CITIZENSHIP Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 979, McManamy	CITY Sherbrooke	STATE & ZIP CODE/COUNTRY Quebec, J1H 2N1, Canada
	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
203	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
	Geneviève Martin	
DATE	DATE 4/7/1	DATE

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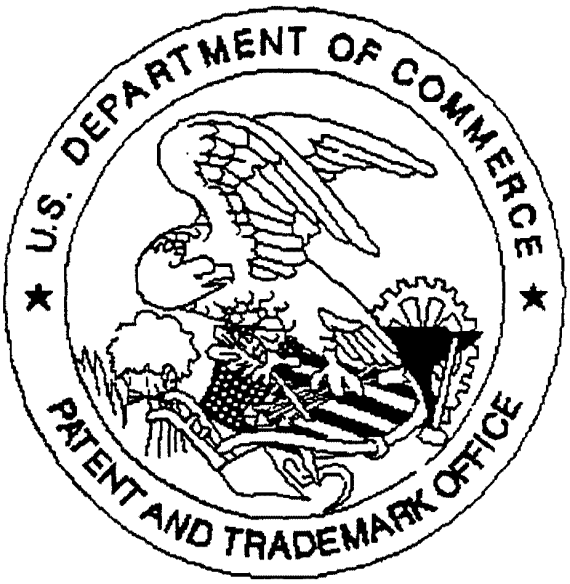

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of BEAUDOIN et al

Application No. 09/830,146 Examiner:

Filed: April 20, 2001 Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

TRANSMITTAL LETTER

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Sir:

Please find enclosed for filing:

- ✓ Response to Notification of Missing Requirements under 35 U.S.C. 371, including copy of Notification of Missing Requirements
- ✓ 2 Signed Declarations and Powers of Attorney
- ✓ Check in the amount of $65.00
- ✓ Return receipt postcard
- ✓ Please charge any deficiencies or credit any overpayments to Deposit Account No. 50-0951.

Respectfully submitted,

Date: 7/23/01

[signature]

Joseph W. Bain
Registration No. 34,290
Mark D. Passler
Registration No. 40,764
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, 4th Floor
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

07/26/2001 MKAYPAGH 00000074 09830146
01 FC:254 65.00 OP

Docket No. 789-47

P1011858;1

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT
United States Patent and Trademark Office
Washington, D.C. 20231
www.uspto.gov

U.S. APPLICATION NO.		FIRST NAMED APPLICANT	ATTY. DOCKET NO.
09/830146		BEAUDOIN	A 789-47

INTERNATIONAL APPLICATION NO.
PCT/CA99/00987

5611

GREGORY A NELSON
AKERMAN SENTERFITT & EIDSON
POST OFFICE BOX 3188
WEST PALM BEACH FL 33402-3188

I.A. FILING DATE	PRIORITY DATE
10/21/99	10/21/98

DATE MAILED: 05/23/01

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

1. The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as ☐ a Designated Office (37 CFR 1.494) ☑ an Elected Office (37 CFR 1.495):

- ☑ U.S. Basic National Fee.
- ☑ Copy of the international application.
- ☑ Oath or Declaration of inventors(s).
- ☐ Copy of Article 19 amendments.
- ☑ Priority Document.
- ☐ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Translation of Annexes to the International Preliminary Examination Report into English.
- ☐ Indication of Small Entity Status.
- ☐ Translation of the international application into English.
- ☐ Translation of Article 19 amendments into English.
- ☐ Other:

2. ☑ Applicant has requested early processing under 35 U.S.C. 371(f) but has not filed the following indicated items and/or the indicated items in paragraph 3 below. The Basic National Fee and the copy of the international application must be filed prior to 20 or 30 months from the priority date to avoid abandonment.

- ☐ U.S. Basic National Fee.
- ☐ Copy of the international application.

3. The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- ☐ a. Translation of the application into English. A processing fee will be required if submitted later than the appropriate 20 or 30 months from the priority date.
  - ☐ The current translation is defective for the reasons indicated on the attached Notice of Defective Translation.
- ☐ b. Processing fee for providing the translation of the application and/or the Annexes later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(f)).
- ☑ c. Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), properly identifying the application (preferably by the International application number and international filing date). A surcharge will be required if submitted later than the appropriate 20 or 30 months from the priority date.
  - ☑ The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) for the reasons indicated on the attached PCT/DO/EO/917.
- ☑ d. Surcharge for providing the oath or declaration later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(e)).

4. Additional claim fees of $_____ as a ☐ large entity ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due (37 CFR 1.492(g)). See attached PTO-875.

5. ☐ Applicant has not submitted the required sequence listing pursuant to 37 CFR 1.821-1.825. See attached PCT/DO/EO/920.

ALL OF THE ITEMS SET FORTH IN 3(a)-3(d), 4 AND 5 ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 22 OR 32 MONTHS (where 37 CFR 1.495 applies) FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

6. If box 3a or 3c is checked, a translation of the Annexes MUST be submitted no later than the time period set above or the Annexes will be cancelled. A processing fee will be required if submitted later than 20 or 30 months from the priority date.

7. ☐ The Article 19 amendments are cancelled since a translation was not provided by the appropriate 20 (37 CFR 1.494(d)) or 30 (37 CFR 1.495(d)) months from the priority date.

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

A copy of this notice MUST be returned with this response.

Enclosed: ☑ PCT/DO/EO/917 ☐ Notice of Defective Translation
☐ PTO-875 ☐ PCT/DO/EO/920

Lamont Hunt r, Paralegal
Telephone: 703.305-3686

FORM PCT/DO/EO/905 (March 2001)

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT
United States Patent and Trademark Office
Washington, D.C. 20231
www.uspto.gov

U.S. APPLICATION NO.		FIRST NAMED APPLICANT	ATTY. DOCKET NO.
09/830146		BEAUDOIN	

INTERNATIONAL APPLICATION NO. PCT/CA99/00847

5611

GREGORY A NELSON
AKERMAN SENTERFITT & EIDSON
POST OFFICE BOX 3188
WEST PALM BEACH FL 33402-3188

I.A. FILING DATE 10/21/98 PRIORITY DATE 05/23/01

DATE MAILED 11/21/99

NOTIFICATION OF A DEFECTIVE OATH OR DECLARATION

This application fails to contain an oath or declaration acceptable under 35 U.S.C. 371(c)(4) for entry into the national stage in the United States of America. The period within which to correct the deficiency noted below and avoid abandonment is set in the accompanying Notification.

A new oath or declaration, properly identifying this application (preferably by the international application number and international filing date) is required. The oath or declaration does not comply with 37 CFR 1.497(a),(b) and (f) in that it:

1. ☑ is not executed in accordance with either 37 CFR 1.66 or 37 CFR 1.68.
2. ☐ does not identify the application to which it is directed.
3. ☐ does not identify the inventor(s).
4. ☐ does not identify the citizenship of each inventor.
5. ☐ does not state that the person making the oath or declaration believes the named inventor or inventors to be the original and first inventor or inventors of the subject matter which is claimed and for which a patent is sought.

FAILURE TO SUBMIT AN OATH OR DECLARATION IN COMPLIANCE WITH 37 CFR 1.497(a) AND (b), AND 1.497(d) WHERE APPROPRIATE, WITHIN THE TIME PERIOD SET WILL RESULT IN FAILURE TO ENTER THE NATIONAL STAGE AND THE ABANDONMENT OF THE APPLICATION.

Additionally, the oath or declaration does not comply with 37 CFR 1.63 in that it:

1. ☐ does not identify the mailing address of each inventor. If the residence is different from the mailing address, then the city and state or city and foreign country of residence of each inventor must also be given.

2. ☐ does not state that the person making the oath or declaration:

   a. ☐ has reviewed and understands the contents of the application, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

   b. ☐ acknowledges the duty to disclose to the Office all information known to the person to be material to patentability as defined in 37 CFR 1.56.

3. ☐ does not identify the foreign application for patent or inventor's certificate for which a claim for priority is made pursuant to 37 CFR 1.55, and any foreign application having a filing date before that of the application on which priority is claimed, by specifying the application serial number, country, day, month, and year of its filing.

Lamont Hunter, Paral gal

Telephone: 703 305-3686

FORM PCT/DO/EO/917 (March 2001)

09/830146

Subclass	ISSUE CLASSIFICATION
Class	

FILED UNDER 35 U.S.C. 371

PATENT NUMBER

U.S. UTILITY Patent Application

O.I.P.E.	PATENT DATE
SCANNED 12C3 Q.A. CS 3	

APPLICATION NO.	CONT/PRIOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
		435	320+	1651	W...+2

APPLICANTS

TITLE

PTO-2040
12/99

ISSUING CLASSIFICATION

ORIGINAL:		CROSS REFERENCE(S)	
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)
INTERNATIONAL CLASSIFICATION			
			☐ Continued on Issue Slip Inside File Jacket

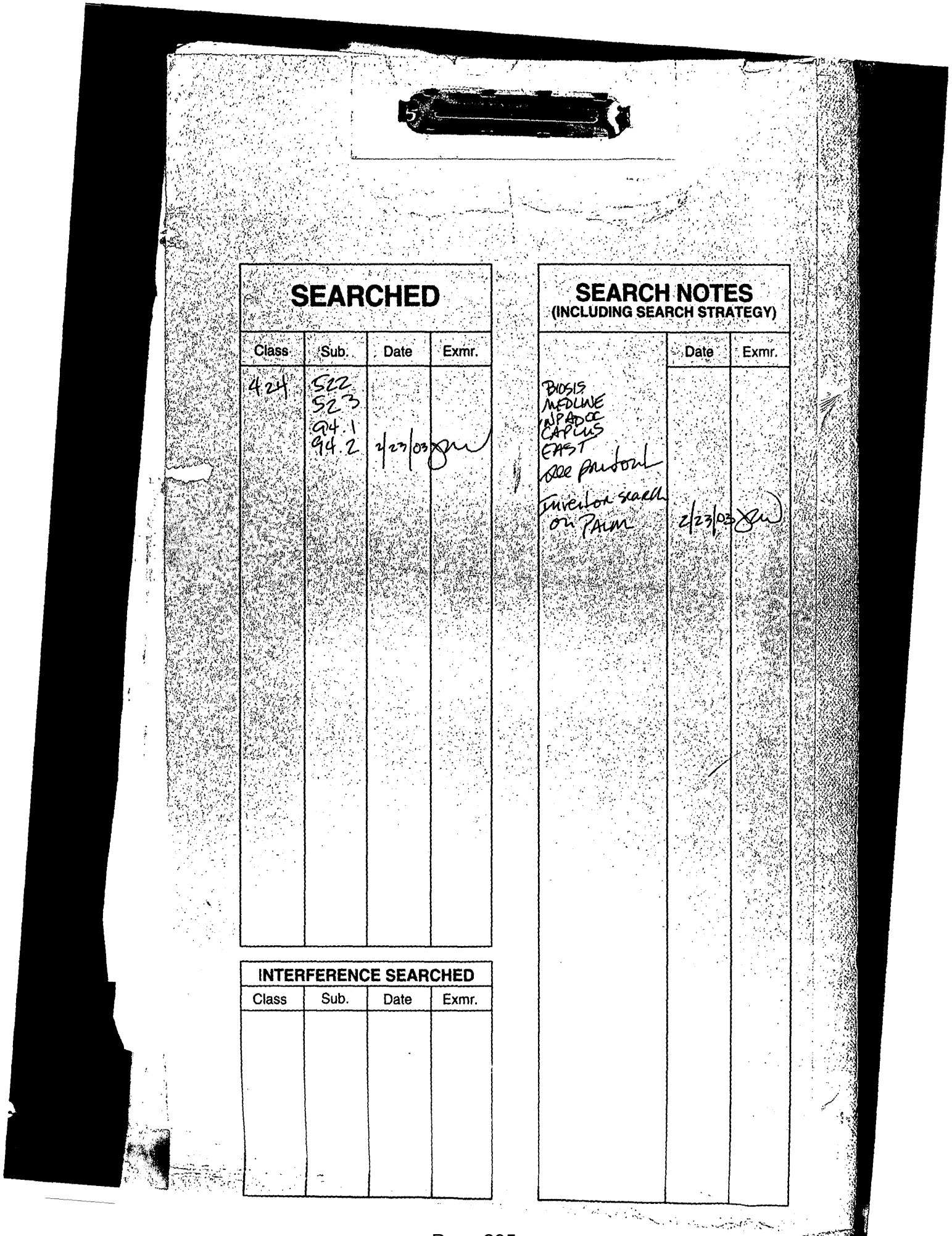
☐ TERMINAL DISCLAIMER	DRAWINGS			CLAIMS ALLOWED	
	Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
☐ The term of this patent subsequent to _____ (date) has been disclaimed.	_____ (Assistant Examiner)		_____ (Date)	NOTICE OF ALLOWANCE MAILED	
☐ The term of this patent shall not extend beyond the expiration date of U.S Patent. No. _____	_____ (Primary Examiner)		_____ (Date)	ISSUE FEE	
				Amount Due	Date Paid
☐ The terminal ____months of this patent have been disclaimed.	_____ (Legal Instruments Examiner)		_____ (Date)	ISSUE BATCH NUMBER	

WARNING:

The information disclosed herein may be restricted. Unauthorized disclosure may be prohibited by the United States Code Title 35, Sections 122, 181 and 368. Possession outside the U.S. Patent & Trademark Office is restricted to authorized employees and contractors only.

Form PTO-436A
(Rev. 6/99)

FILED WITH: ☐ DISK (CRF) ☐ FICHE ☐ CD-ROM
(Attached in pocket on right inside flap)



SEARCHED

Class	Sub.	Date	Exmr.
424	522 523 94.1 94.2	2/23/03	

SEARCH NOTES
(INCLUDING SEARCH STRATEGY)

	Date	Exmr.
BIOSIS MEDLINE WPADOC CAPLUS EAST See previous Inventor search on PALM	2/23/03	

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.

09/830146

ISSUE SLIP STAPLE AREA (for additional cross references)

POSITION	INITIALS	ID NO.	DATE
FEE DETERMINATION			
O.I.P.E. CLASSIFIER	[illegible]	45	5/14
FORMALITY REVIEW			
RESPONSE FORMALITY REVIEW	[illegible]		8-1-01

INDEX OF CLAIMS

Symbol	Meaning	Symbol	Meaning
✓	Rejected	N	Non-elected
=	Allowed	I	Interference
– (Through numeral)	Canceled	A	Appeal
÷	Restricted	O	Objected

Claim Final	Claim Original	Claim Final	Claim Original	Claim Final	Claim Original
	1		51		101
	2		52		102
	3		53		103
	4		54		104
	5		55		105
	6		56		106
	7		57		107
	8		58		108
	9		59		109
	10		60		110
	11		61		111
	12		62		112
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	43		93		143
	44		94		144
	45		95		145
	46		96		146
	47		97		147
	48		98		148
	49		99		149
	50		100		150

If more than 150 claims or 10 actions staple additional sheet here

04-23

Form PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 78[redacted]7
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		US APPLICATION NO. (if known, see 37 CFR 1.5) 09/830146
INTERNATIONAL APPLICATION NO. PCT/CA99/00987 APR 2 0 2001	INTERNATIONAL FILING DATE 21 October 1999	PRIORITY DATE CLAIMED 21 October 1998
TITLE OF INVENTION METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES		
APPLICANT(S) FOR DO/EO/US BEAUDOIN, Adrien; MARTIN, Geneviève		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) [unsigned].

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [x] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [ ] Other items or information:

EXPRESS MAIL LABEL NO. EL 740156190 US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/830146	INTERNATIONAL APPLICATION NO. PCT/CA99/00987	ATTORNEY'S DOCKET NUMBER 789-47

17. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total claims	44	-20 =	14	X **$18.00**	$252.00	
Independent claims	5	-3 =	2	X **$80.00**	$160.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =					$1272.00	
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					$636.00	
SUBTOTAL =					$636.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.429(f)). +					$	
TOTAL NATIONAL FEE =					$636.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					$	
TOTAL FEES ENCLOSED =					$636.00	
					Amount to be: refunded	$
					Charged	$

a. [X] A check in the amount of $ 636.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0951 in the amount of $ 0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0951. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Gregory A. Nelson
Akerman, Senterfitt & Eidson, P.A.
Post Office Box 3188
West Palm Beach, FL 33402-3188

SIGNATURE

Joseph W. Bain
NAME

34,290
REGISTRATION NUMBER

FORM PTO-1390 (REV 11-98) page 2 of 2

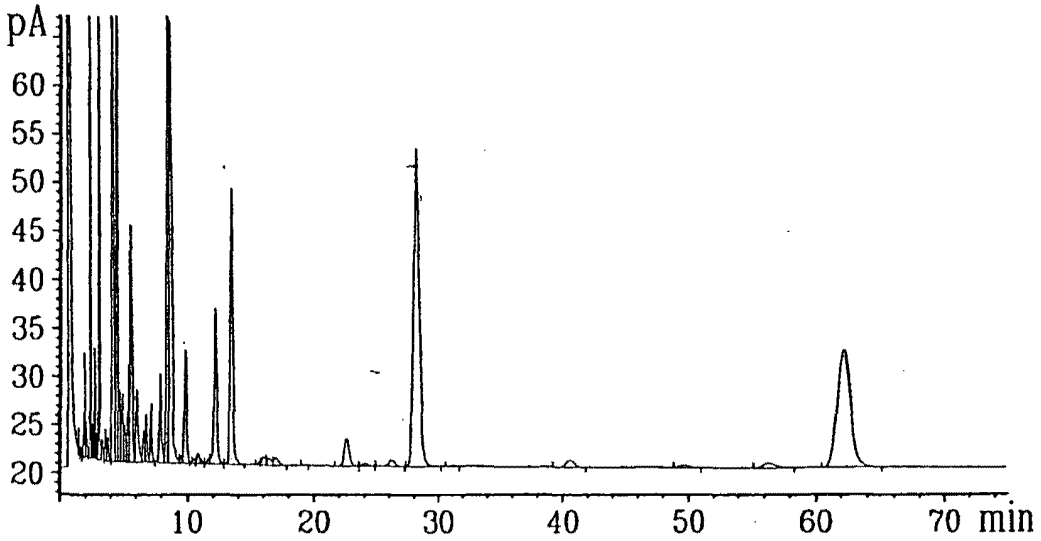


Fig. 1

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	

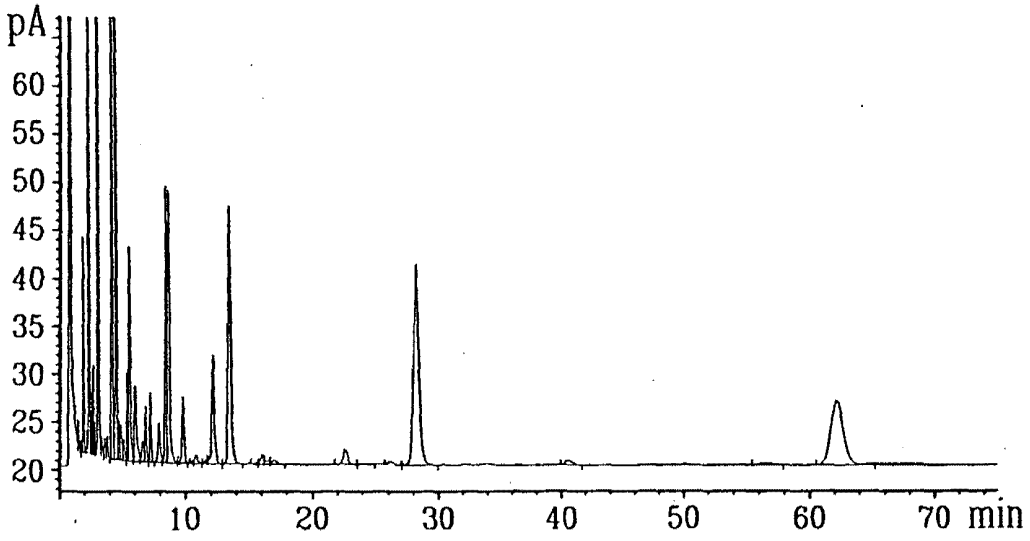


Fig. 2

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

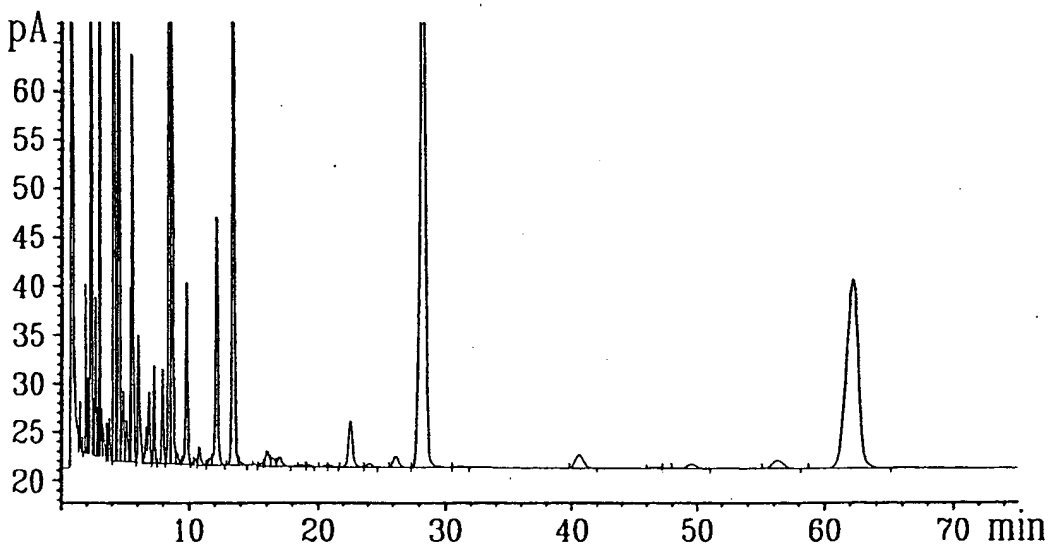


Fig 3

1.216	4.520 - 16:1	13.457
1.262	4.683	13.943
1.454 - 12:0	4.884 - 16:1tr	15.053
1.624	5.030	15.572 - 20:0
1.811	5.111	16.016
1.875	5.420	16.486 - 20:1
2.016	5.561	16.999 - 20:1(cis11)
2.054	6.031	18.762
2.174	6.642	19.303 - 20:2
2.330 - 14:0	6.868	20.474
2.505	7.226	21.027 - 20:3
2.589 - 14:1	7.908 - 18:0	22.575 - 20:4 (6,10,14,18)
2.679	8.444 - 18:1	24.071
2.799	8.639 - 18:1tr	26.215
2.854	9.005	28.333
2.981	9.536	31.180
3.074 - std 15:0	9.788 - 18:2	40.560
3.304	10.267	46.595
3.580	10.481	49.513
3.804	10.807	56.292
4.169 - 16:0	11.626	62.250
4.296	12.140 - 18:3	

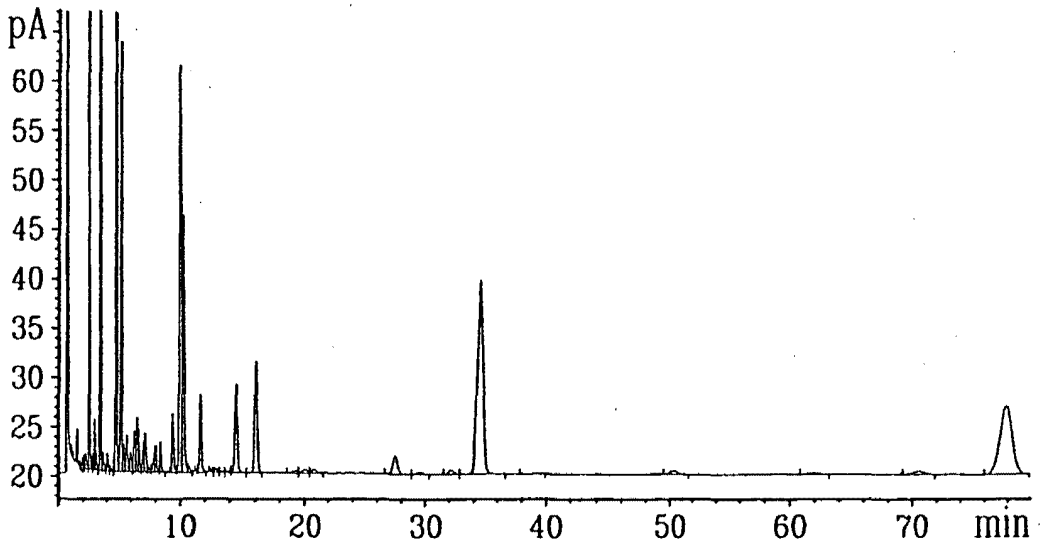


FIG. 4

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	

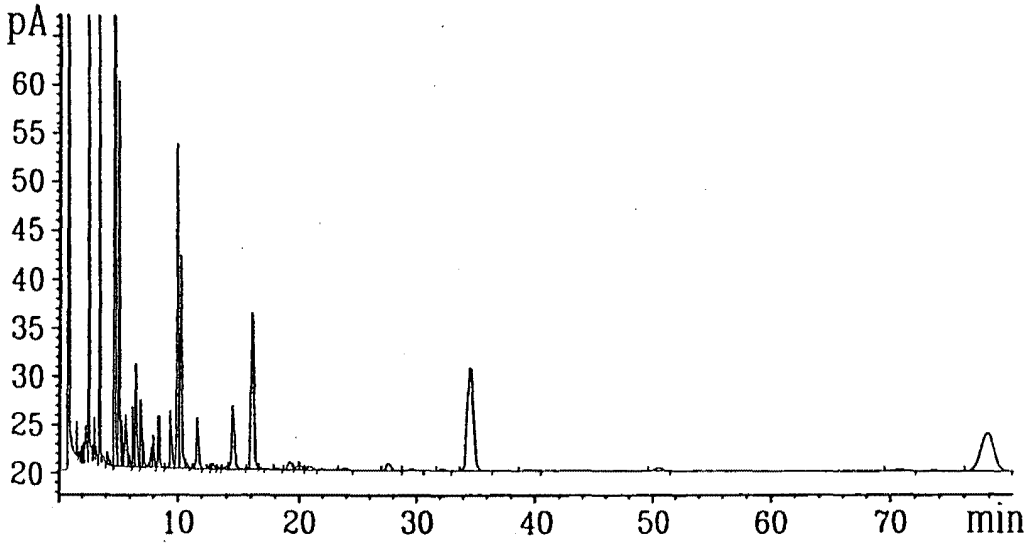


Fig. 5

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	

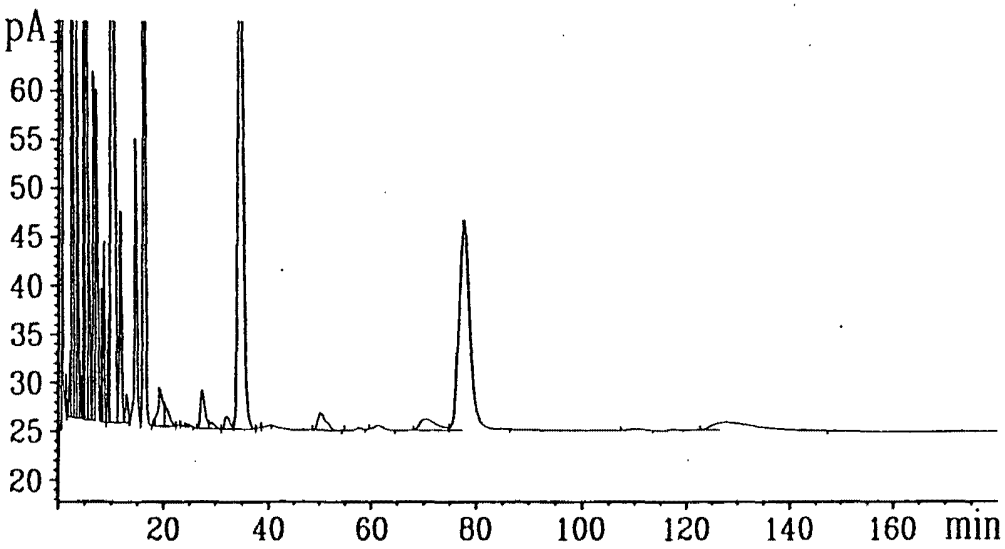


Fig. 6

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	

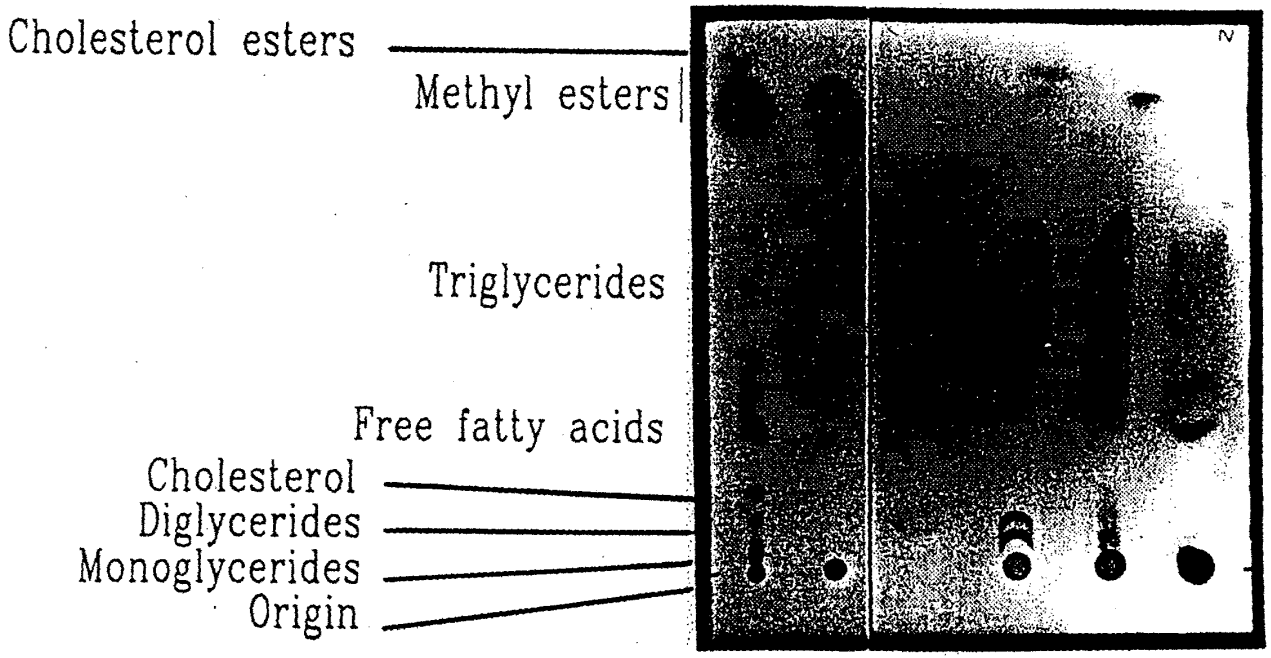


FIG. 7

FIG. 8

8/20

09/830146

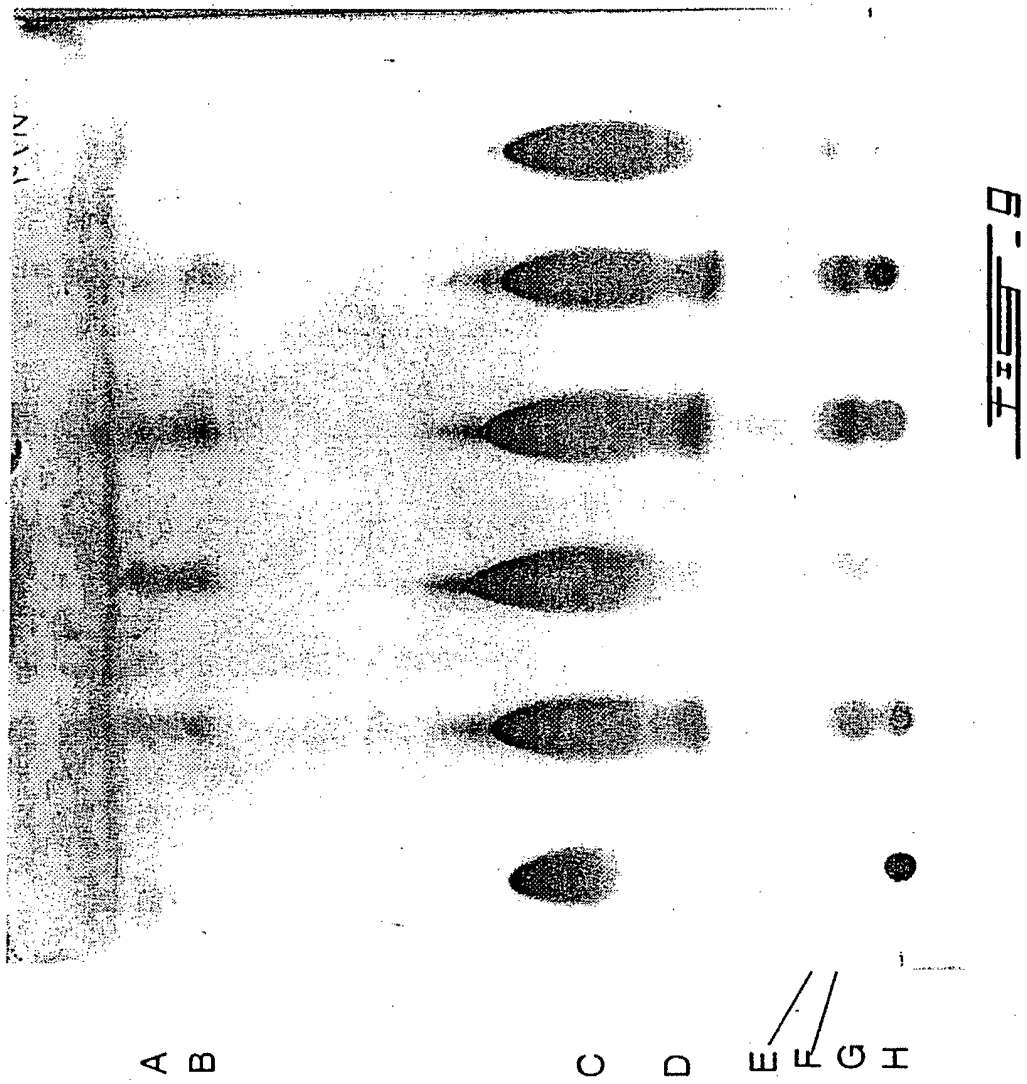


FIG. 8

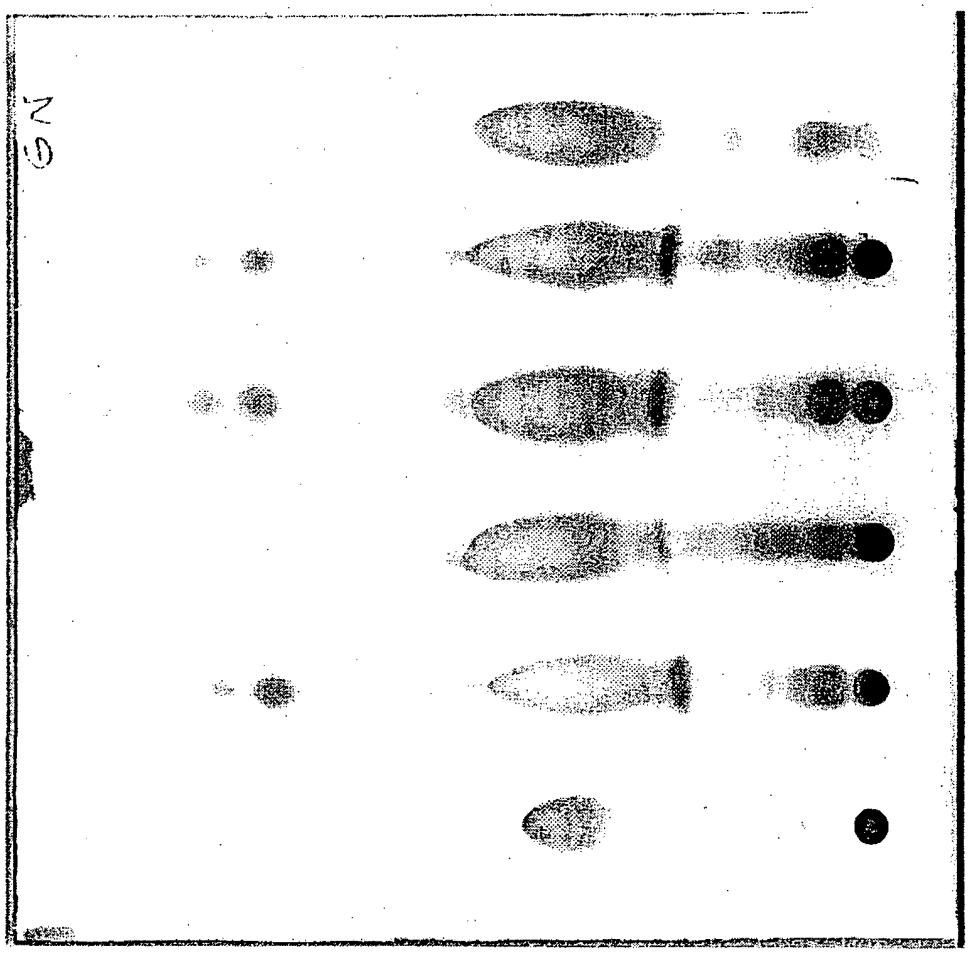


FIG. 10

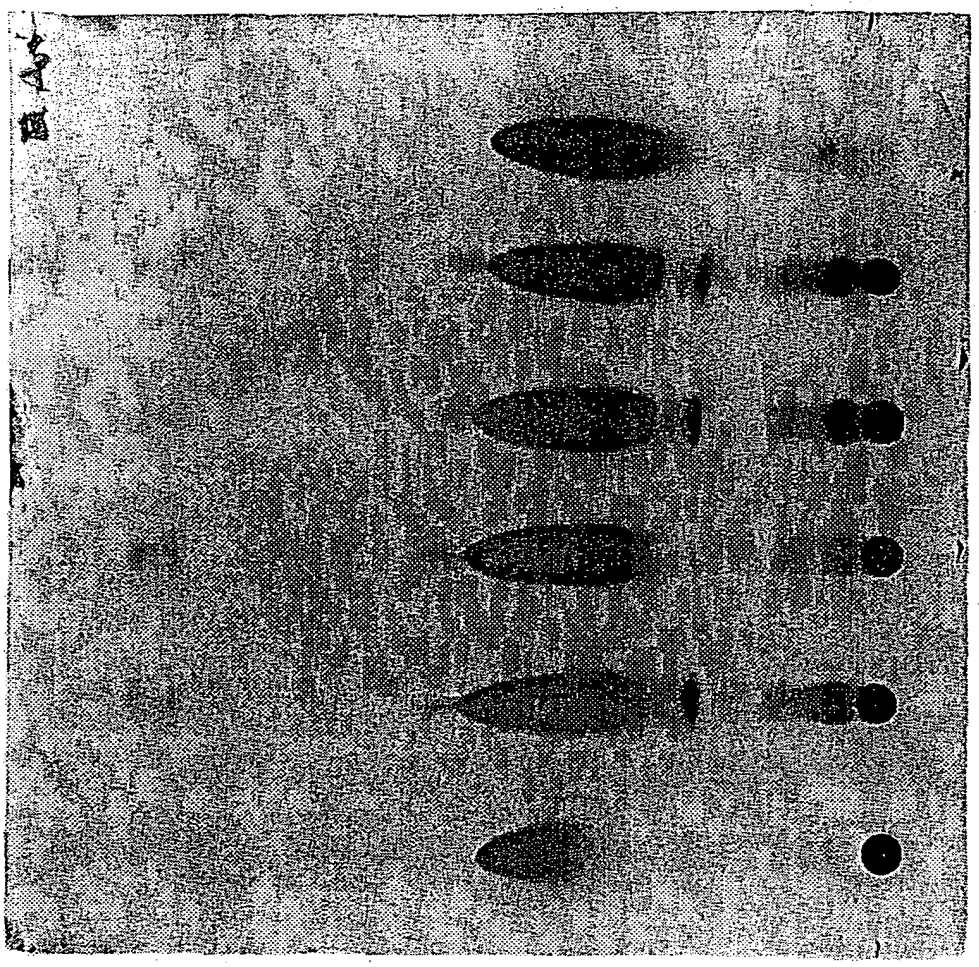


Fig. 11

A B C D E F G H

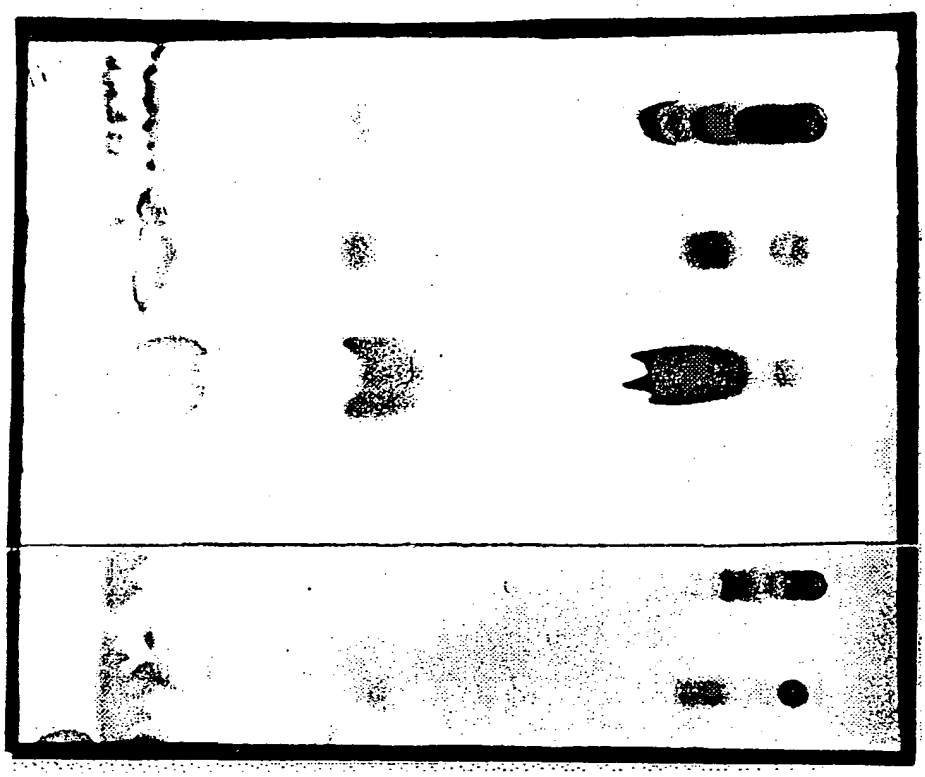

Neutral lipids
Cephalin
Lecithin
Sphingomyelin
Lysolecithin
Origin

Fig. 12

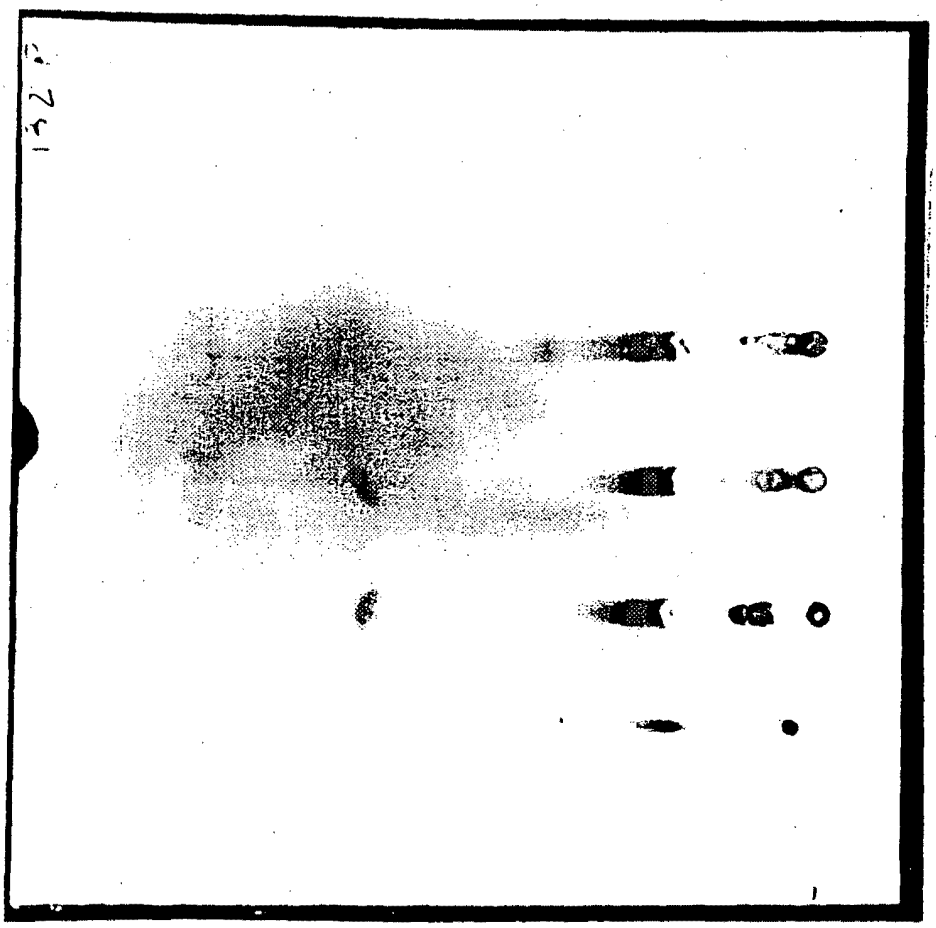


FIG. 13

Neutral lipids

Cephalin

Lecithin

Sphingomyelin

Lysolecithin

Origin

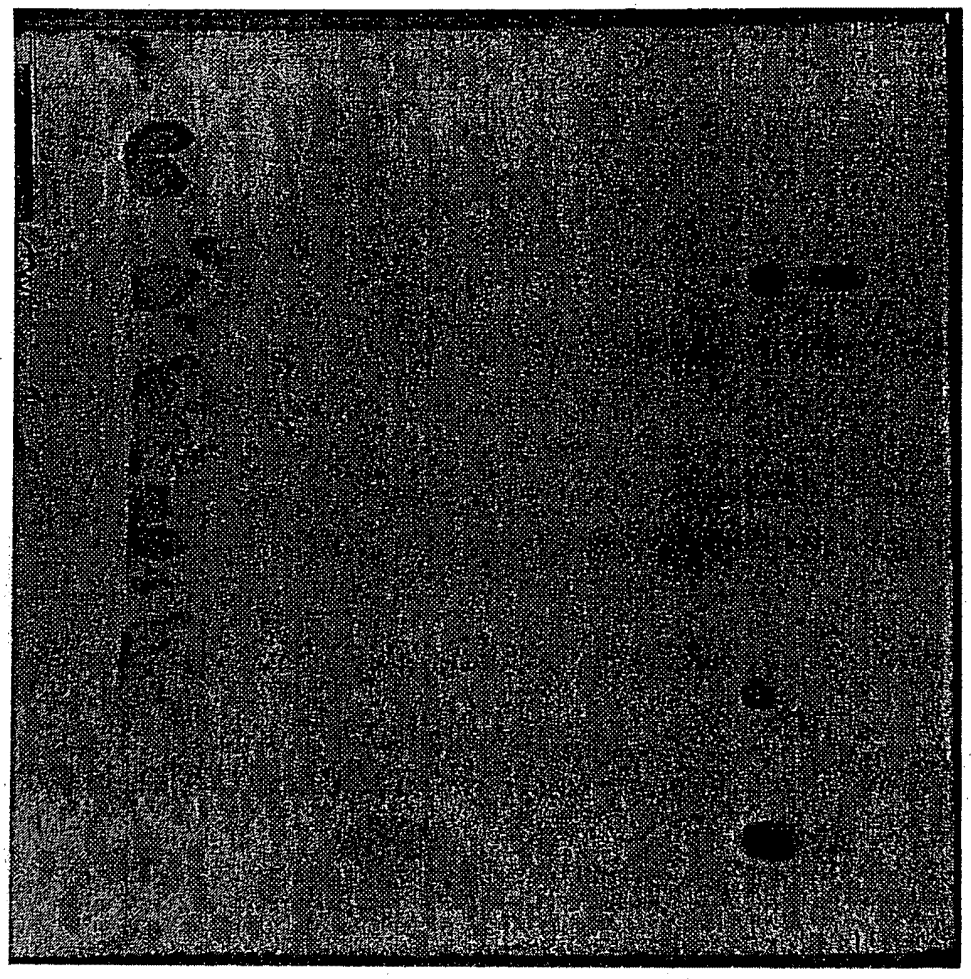


Fig-14

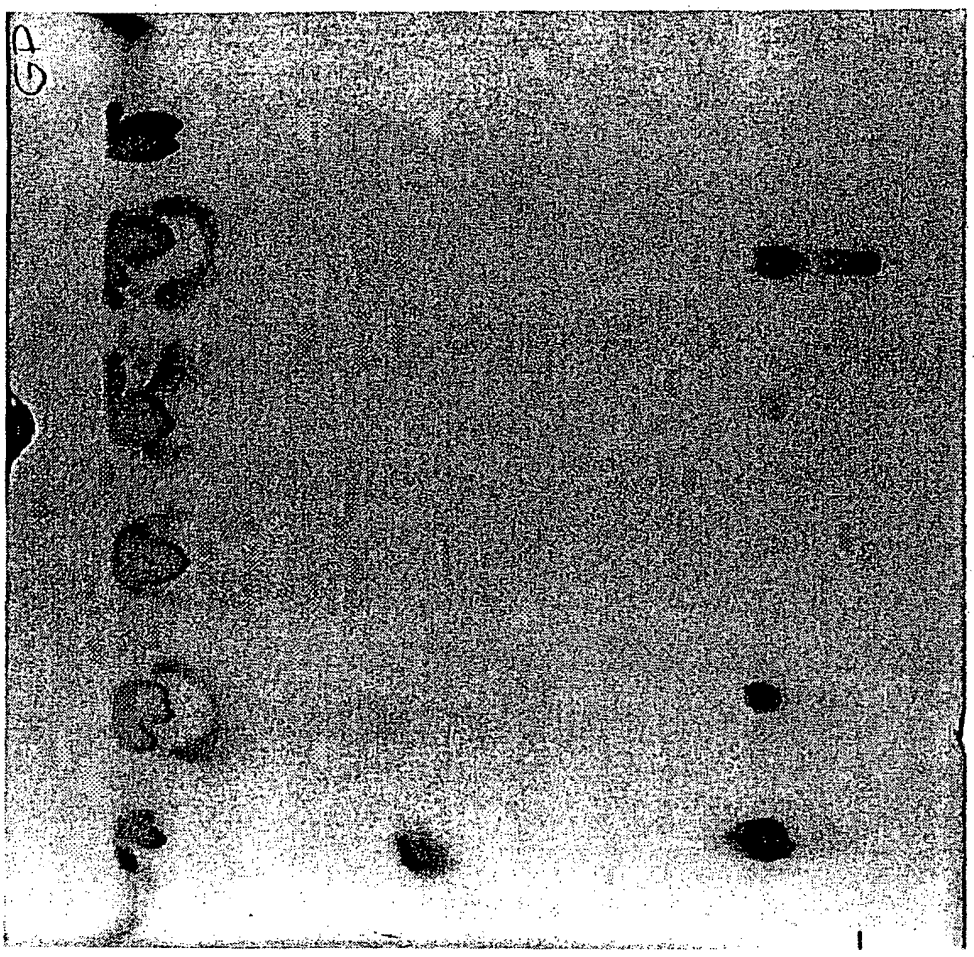


Fig-15

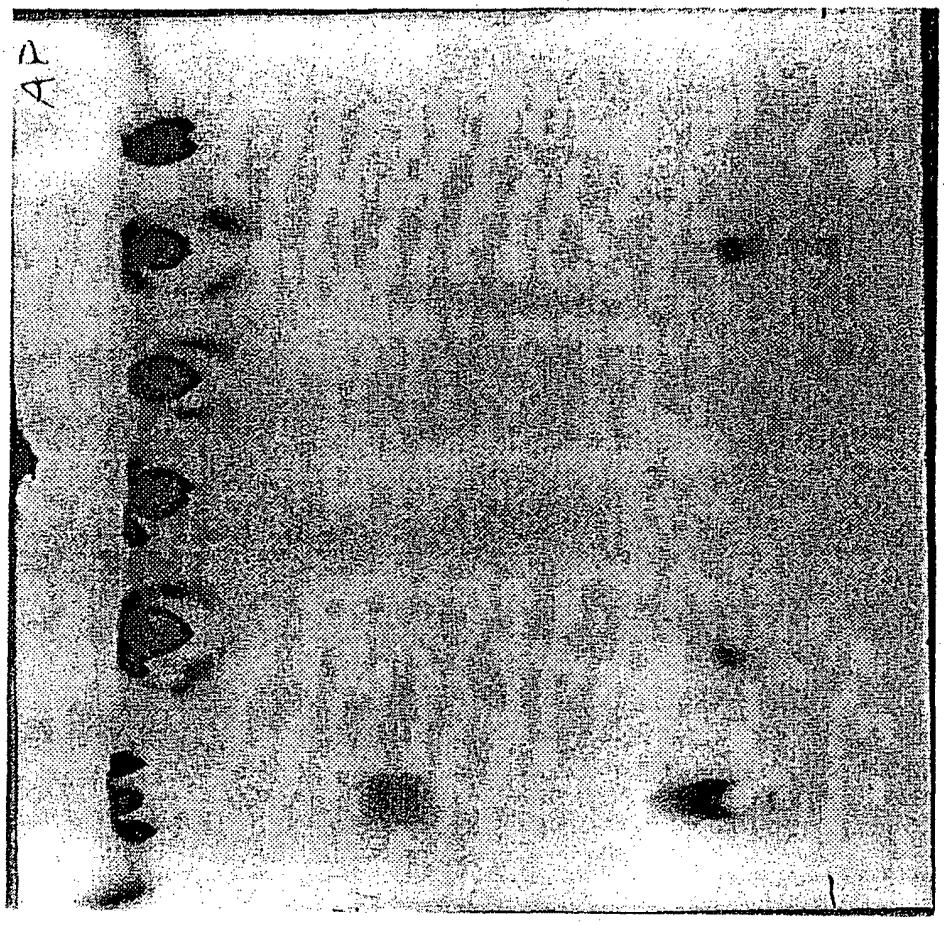


Fig-16

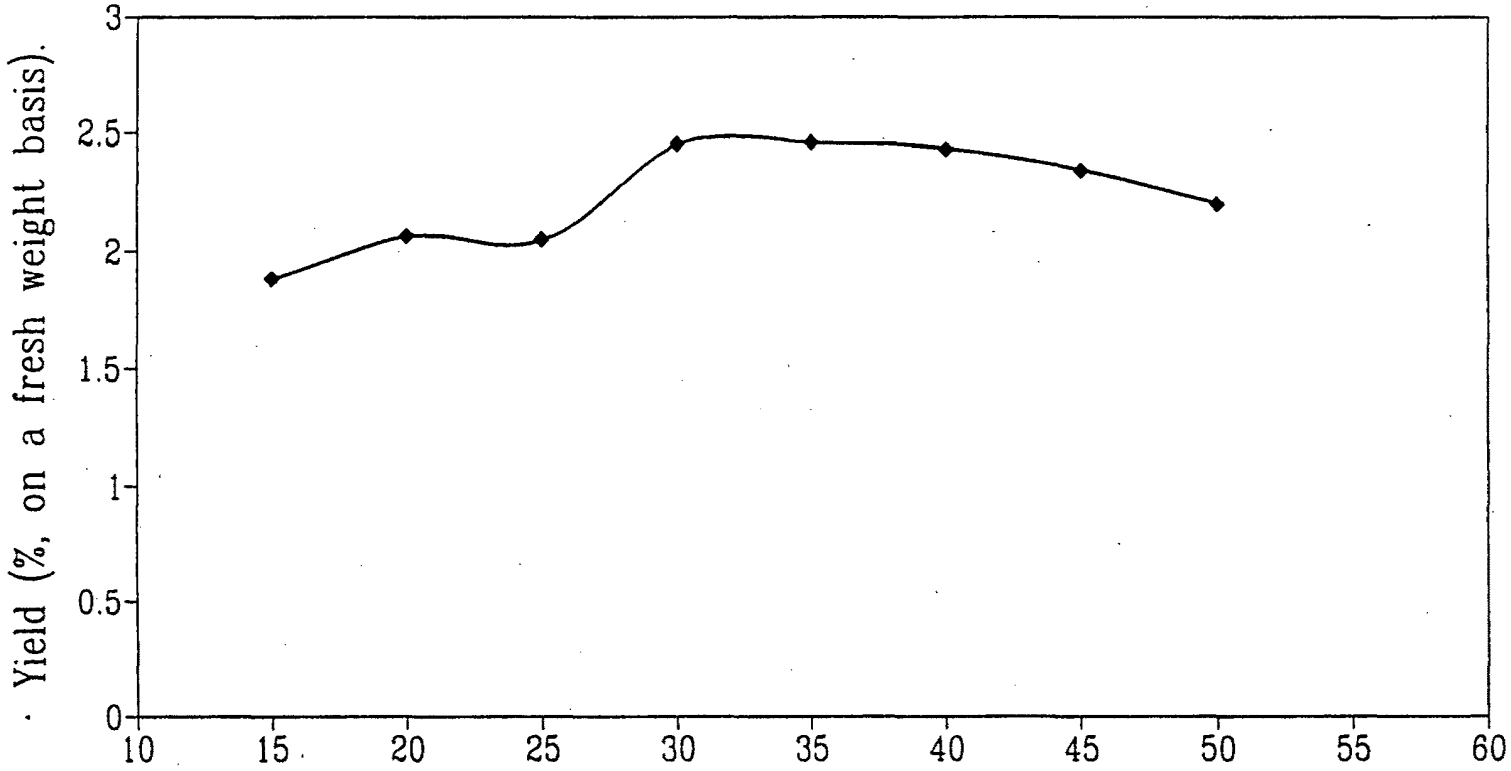

Yield (%, on a fresh weight basis).
3
2.5
2
1.5
1
0.5
0
10
15
20
25
30
35
40
45
50
55
60
Volume of acetone (mL).
Incubation time of 2 h.
Determinations in triplicates (variation less than 5 %).

FIG. 17

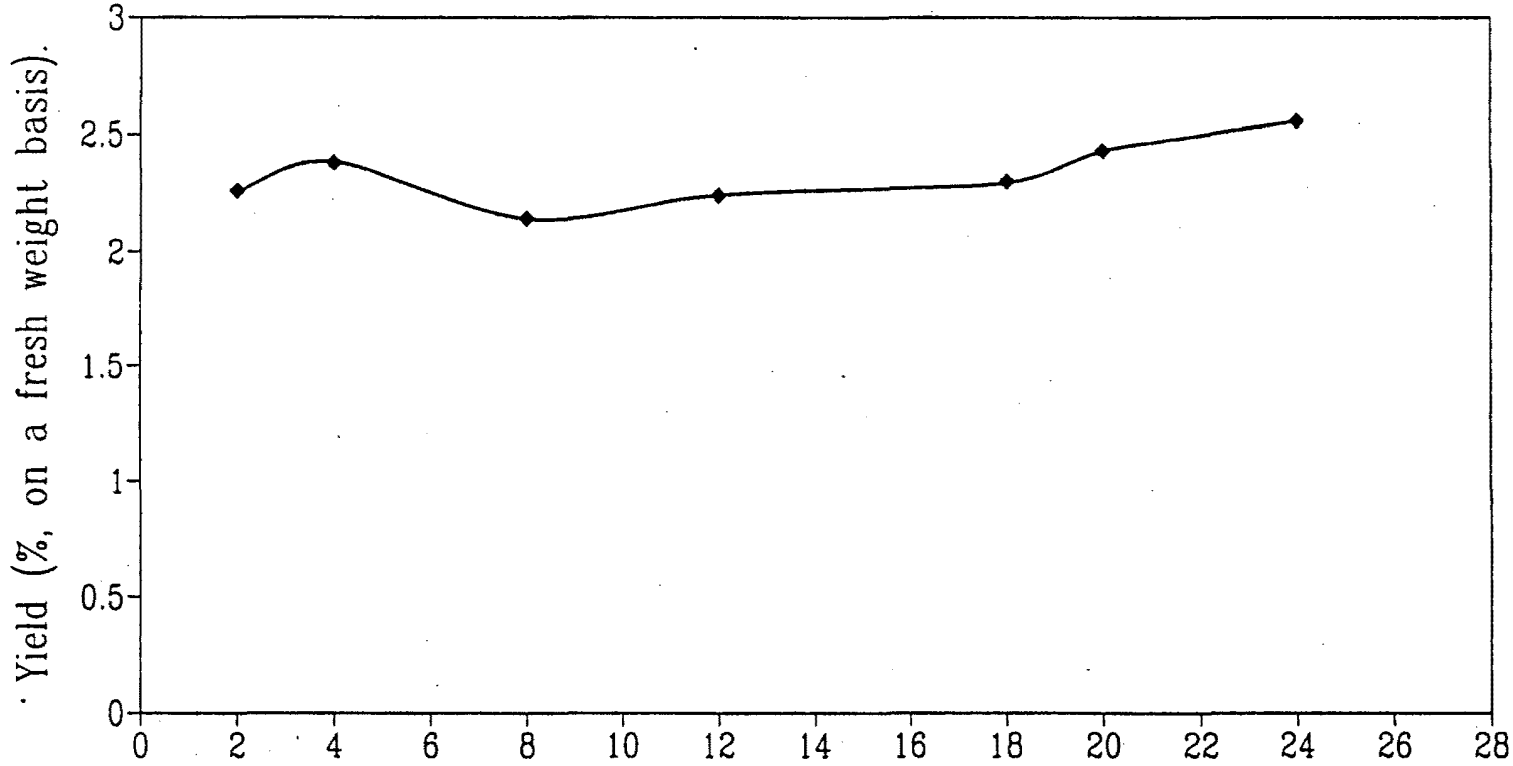

Yield (%, on a fresh weight basis).
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Incubation time in acetone (h).
Sample-acetone ratio of 1:9 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 18

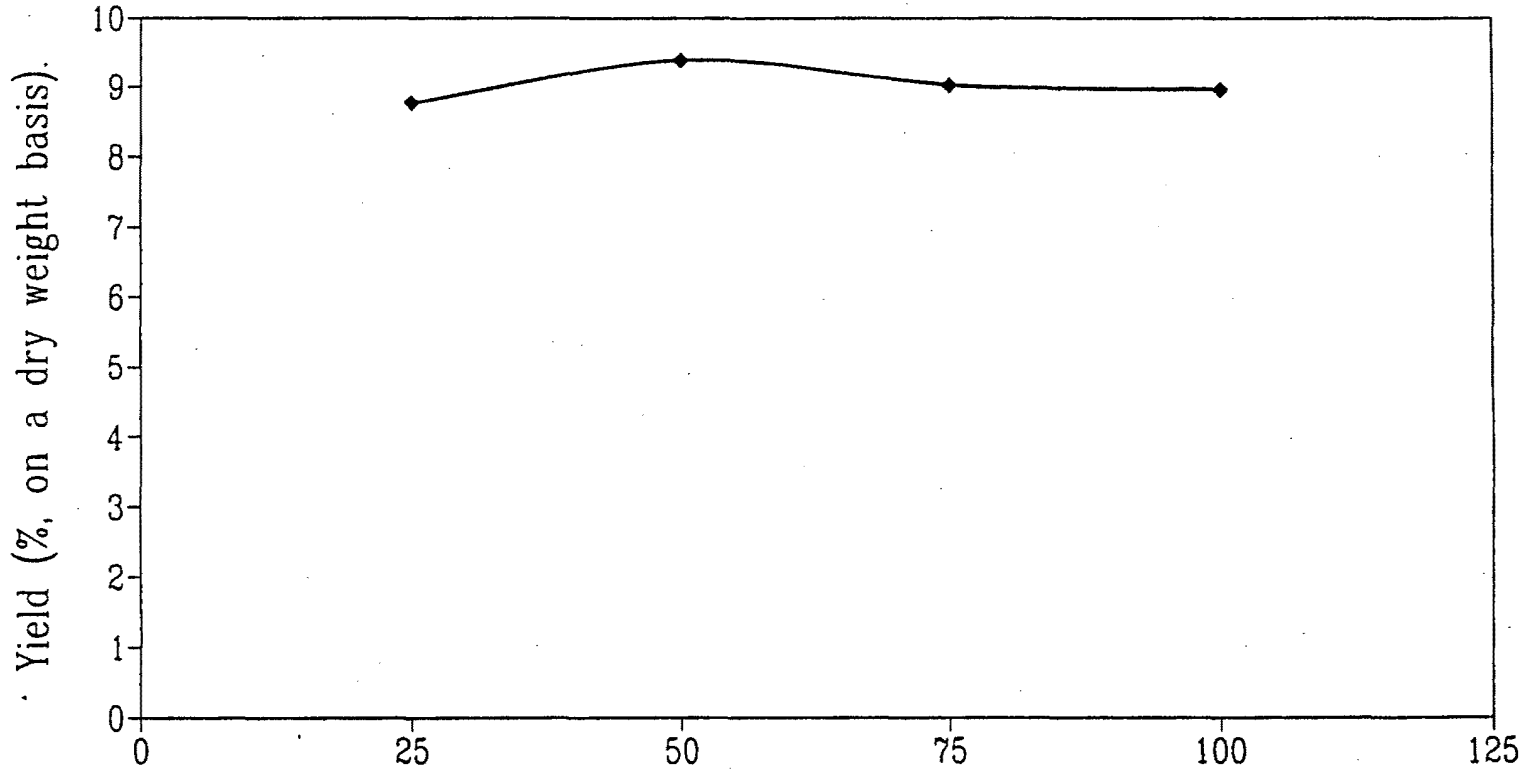

Yield (%, on a dry weight basis).
10
9
8
7
6
5
4
3
2
1
0
0
25
50
75
100
125
Volume of ethanol (mL).
Incubation time of 30 min.
Determinations in triplicates (variation less than 5 %).

Fig. 19

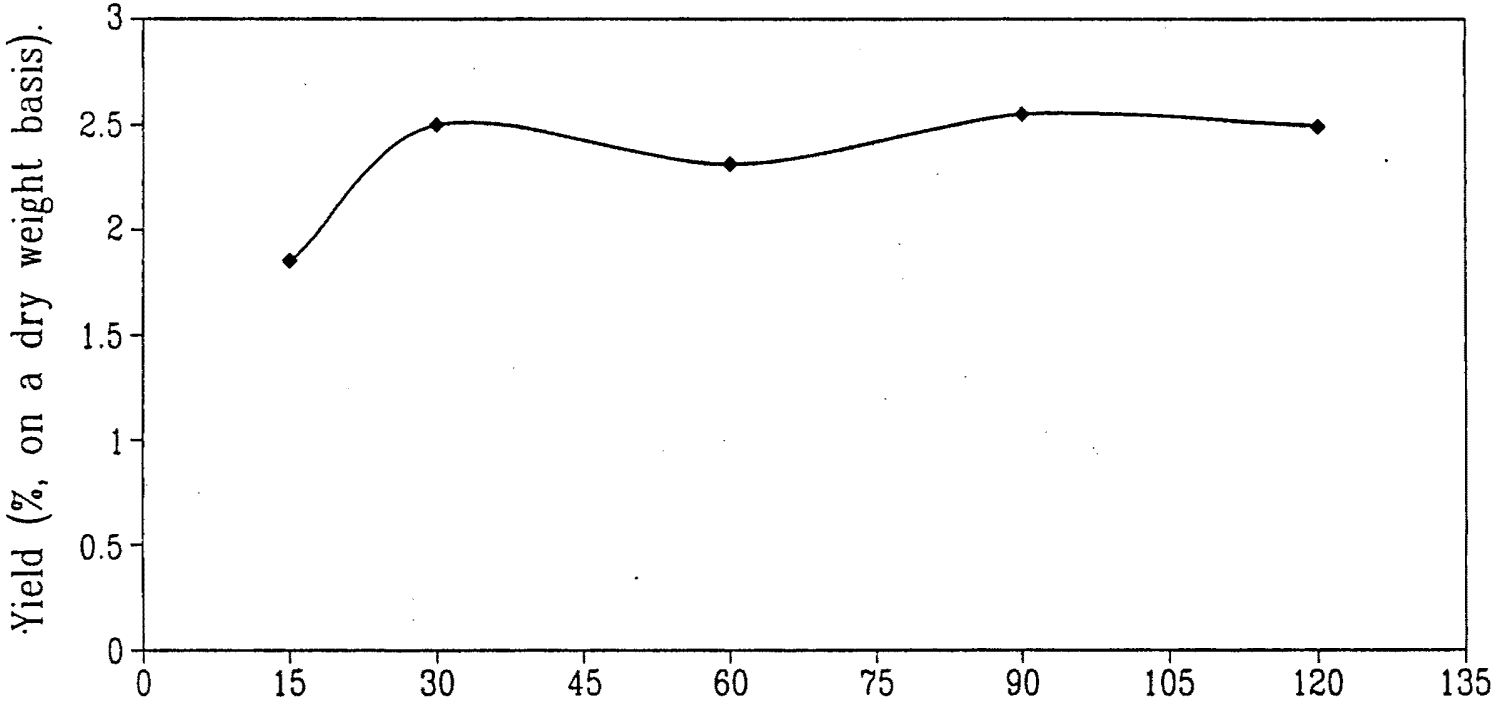

Yield (%, on a dry weight basis).
3
2.5
2
1.5
1
0.5
0
0
15
30
45
60
75
90
105
120
135
Incubation time in ethanol (min).
Sample-ethanol ratio of 1:4 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 20

TITLE OF THE INVENTION

METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

FIELD OF THE INVENTION

This invention relates to the extraction of lipid fractions from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals. More specifically, this invention relates to an improved method of extracting lipid fractions by dehydration with solvents and recovering a solid residue rich in active enzymes.

BACKGROUND OF THE INVENTION

Lipid fractions obtained from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals have various applications:

Medical applications

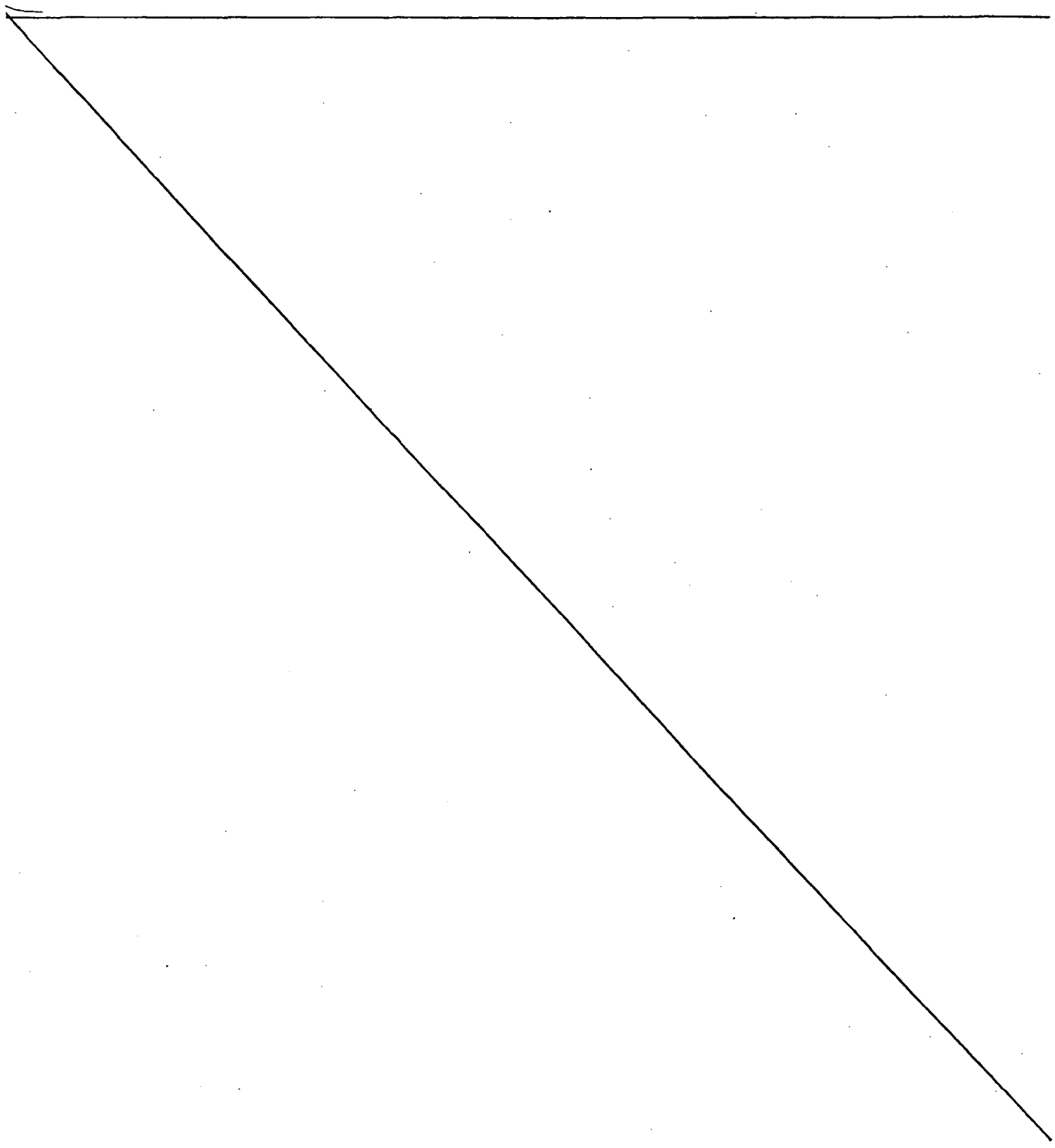
Marine and aquatic animal oils and fractions thereof contain various therapeutic agents. For example, it is reported that various marine and aquatic animal oils have anti-inflammatory properties. Marine and aquatic animal oils are also reported as helpful in reducing the incidence of cardiovascular disease. Also, some marine and aquatic animal oils are reported as suppressing the development of certain forms of lupus and renal diseases. As a further example, krill may be used as a source of enzymes for debridement of ulcers and wounds or to facilitate food digestion. Also marine and aquatic oils contain various antioxidants, which may have potential therapeutic properties.

Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. These fatty acids are essential for proper development of the brain and the eye. Marine and aquatic animal oils are also rich in liposoluble vitamins A, D and E and carotenoids.

Cosmetics

Various marine and aquatic animal oils are used for the production of moisturizing creams.



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 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*" 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 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 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.

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)

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)

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 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 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 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 (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.

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 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 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 reference for measuring efficiency of the extraction process. Another comparison has been made with a technique using hexane as the extraction solvent. Lipid recovery

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 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 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) including some modifications to the original technique: 2h at 65°C 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.

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. The same 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, astaxanthin 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.

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 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).

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

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.

Tables 12 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 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 14 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 12 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 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 12, 13, 14, 15, 16 and 17. It is noteworthy to mention that in Table 17, the carotenoids content was significantly high as measured in terms of two carotenoids namely astaxanthin and canthaxanthin. Indeed, duplicates analyzes revealed values of 92 to 124 µg/g of lipid fraction for astaxanthin 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 astaxanthin at least 75 and preferably at least 90 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 (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.

Table 18 shows the best mode of the method in accordance with the present invention for lipid extraction of aquatic animal tissues.

Table 19 shows that the enzyme activity of the solid fraction is maintained following 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 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].

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.

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 (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)

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

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 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.

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.

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.

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.

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.

BIBLIOGRAPHY

Bowyer, D.E., Leat, W.M.F., Howard, A.N. and Gresham, G.A. 1962. The determination of the fatty acid composition of serum lipids separated by thin-layer chromatography; and a comparison with column chromatography. BBA. 70: 423-431.

Chandrasekar, B., Troyer, D.A., Venkatraman, J.T. and Fernandes, G. 1996. Tissue specific regulation of transforming growth factor beta by omega-3 lipid-rich krill oil in autoimmune murine lupus. Nutr Res. 16(3): 489-503.

Christensen, M.S., Hoy, C-E. and Redgrave, T.G. 1994. Lymphatic absorption of *n*-3 polyunsaturated fatty acids from marine oils with different intramolecular fatty acid distributions. BBA. 1215: 198-204.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. 1983. Spectrophotometric assay using *o*- Phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci. 66: 1219-1227.

Difco laboratories. 1984. Difco Manual Dehydrated Culture Media and Reagents for Microbiology. 10th ed. Detroit.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem. 226: 497-509.

Goodman Gilman, A., Goodman, L.L. and Gilman, A. 1980. The Pharmacological Basis of Therapeutics. 6th ed. Collier Macmillan Canada ltd, Toronto.

Harwood, H.J. and Geyer, R.P. 1964. Biology Data Book. The Federation of American Societies for Experimental Biology, Washington.

Hellgren, L., Karlstam, B., Mohr, V. and Vincent, J. 1991. Krill enzymes. A new concept for efficient debridement of necrotic ulcers. Int J Dermatol. 30(2): 102-103

Plummer, D.T. 1987. An introduction to practical biochemistry. 3th ed. McGraw-Hill Book Company, London.

Rawn, J.D. 1990. Traité de biochimie. De Boeck-Wesmael, Bruxelles.

Runge, J.A. and Joly, P. 1994. Rapport sur l'état des invertébrés en 1994: 7:0 Zooplancton (Euphausiacés et *Calanus*) de l'Estuaire et du Golfe du Saint-Laurent.

Sargent, J.R. 1997. Fish oils and human diet. Br J Nutr.78 Suppl 1: S5-S13.

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

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

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
7-	acetone [a]	2,15		
	t-butanol [c]	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]	2,28		
	ethyl acetate [b]	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**	

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.

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

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.

TABLE 6. EXTRACTION OF FRESH FISH LIPIDS (Macker I)

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

[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.

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.

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 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**
2-	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. 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 ™.

TABLE 13. 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 14. 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 14 (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 14 (continu d). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
22:6 n-3 Hexaenes	13,2	6,6
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 15. 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

TABLE 15 (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.

TABLE 16. 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 17. 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.

TABLE 18. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (sugg sted proc dure)

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 19: 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

CLAIMS

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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

(g) recovering the solid contents.

~~4.~~ A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

~~5.~~ A method as in claim 1, wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

~~6.~~ A method as in claim 1 , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

~~7.~~ A method as in claim 1, 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).

~~8.~~ A method as in claim 1, 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 claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

~~10.~~ A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

~~11.~~ A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

~~12.~~ A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

~~14.~~ A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

~~15.~~ A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

13
~~16~~. A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

14
~~17~~. A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

15
~~19~~. A method as in claim 15, wherein during step (a), the animal material is homogenized.

16
~~20~~. A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

17
~~21~~. A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

18
~~22~~. A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

19
~~23~~. A method as in claim 15, wherein after step (b) and before step (c), the method

additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

~~24~~ 20. A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

~~25~~ 21. A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

~~26~~ 22. A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

~~27~~ 23. A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

~~30~~ 24. A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

~~31~~ 25. 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

whereby lipid fractions are obtained.

26
~~32.~~ A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

27
~~33.~~ A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

28
~~36.~~ A method as in claim 1, wherein the ketone solvent is acetone.

29
~~37~~. A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

30
~~38~~. A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

31
~~39~~. A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

32
~~40~~. A method as in claim 15, wherein said marine and aquatic animal material is viscera.

33
~~41~~. A method as in claim 15, wherein the ketone solvent is acetone.

34
~~42~~. A method as in claim 16, wherein said marine and aquatic animal material is viscera.

35
~~43~~. A method as in claim 16, wherein the animal material is selected from krill and *Calanus.*

36
~~44~~. A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

37
~~45~~. A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

38
~~46~~. A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

39
~~47~~. A method as in claim 1, wherein the solid contents of step (e) is recovered and

consists of a dehydrated residue containing active enzymes.

46
~~48.~~ A method as in claim 31, wherein the ketone solvent is acetone.

41
~~49.~~ A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

42
~~50.~~ A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

43
~~51.~~ A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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2,251,265 21 October 1998 (21.10.98) CA

(71) Applicant *(for all designated States except US)*: UNIVERSITE DE SHERBROOKE [CA/CA]; University Boulevard, Sherbrooke, Quebec J1K 2R1 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BEAUDOIN, Adrien [CA/CA]; 748, boulevard des Vétérans, Rock Forest, Quebec J1N 1Z7 (CA). MARTIN, Geneviève [CA/CA]; 797, McManamy, Sherbrooke, Quebec J1H 2N1 (CA).

(74) Agents: DUBUC, Jean, H. et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, P.O. Box 242, 800 Place Victoria, Montreal, Quebec H4Z 1E9 (CA).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(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.

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
789-47

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

---

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _
on _,
as amended on _ (if applicable).

[x] was filed as a PCT international application number PCT/CA99/00987 on 21 October 1999 as amended under PCT Article 19 on ___ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
CANADA	2,251,265	21 October 1998	[x] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

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U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS			
PCT/CA99/00987	21 October 1999				

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith: J. Rodman Steele, Jr., Registration No. 25,931; Gregory A. Nelson, Registration No. 30,577, Joseph W. Bain, Registration No. 34,290; Robert J. Sacco, Registration No. 35,667; Mark D. Passler, Registration No. 40,764; Stanley A. Kim, Registration No. 42,730; Steven M. Greenberg, Registration No. 44,725; Neil R. Jetter, Registration No. 46,803; Larry G. Brown, Registration No. 45,834; Kevin T. Cuenot, Registration No. 46,283; Pablo Meles, Registration No. 33,739; Raynaldo K. Whitty, Registration No. 47,176; and Barbara S. Kitchell, Registration No. 33,928.

Send Correspondence to:
Akerman, Senterfitt & Eidson, P.A.
Post Office Box 3188
West Palm Beach, FL 33402-3188

Direct Telephone Calls to:
(561) 653-5000

201	FULL NAME OF INVENTOR	FAMILY NAME: BEAUDOIN	FIRST GIVEN NAME: Adrien	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: Rock Forest CAN	STATE OR COUNTRY: Quebec, Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 748, boulevard des Vétérans	CITY: Rock Forest	STATE & ZIP CODE/COUNTRY: Quebec, J1N 1Z7, Canada
202	FULL NAME OF INVENTOR	FAMILY NAME: MARTIN	FIRST GIVEN NAME: Geneviève	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: Sherbrooke	STATE OR COUNTRY: Quebec. Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 979, McManamy	CITY: Sherbrooke	STATE & ZIP CODE/COUNTRY: Quebec, J1H 2N1, Canada
203	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	POST OFFICE ADDRESS:	CITY:	STATE & ZIP CODE/COUNTRY:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
A Beaudoin		
DATE: 31 juillet 2001	DATE	DATE

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
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As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

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---

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _
on _,
as amended on _ (if applicable).

[x] was filed as a PCT international application number PCT/CA99/00987 on 21 October 1999 as amended under PCT Article 19 on __ (if applicable).

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			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

PTC 1391 Rev 10-83 Page 1 of 2 US DEPARTMENT OF COMMERCE Patent and Trademark Office

P1011126;1

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER 789-47

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U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS			
PCT/CA99/00987	21 October 1999				

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	FULL NAME OF INVENTOR	FAMILY NAME: MARTIN	FIRST GIVEN NAME: Geneviève	SECOND GIVEN NAME
202	RESIDENCE & CITIZENSHIP	CITY: Sherbrooke CAN	STATE OR COUNTRY: Quebec, Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 979, McManamy	CITY: Sherbrooke	STATE & ZIP CODE/COUNTRY: Quebec, J1H 2N1, Canada
	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
203	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
	Geneviève Martin	
DATE	DATE 4/7/1	DATE

PTC 1391 Rev 10-83 Page 2 of 2 DEPARTMENT OF COMMERCE Patent and Trademark Office

P1011126;1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of BEAUDOIN et al

Application No. 09/830,146 Examiner:

Filed: April 20, 2001 Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS AND SUBMISSION OF SIGNED DECLARATION

CERTIFICATE UNDER 37 CFR 1.8(a)
I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, Washington, D.C. 20231 on 7/23/01
[signature] Reg. No. 40,764

Box PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

In the above-identified application, Applicants have received a Notification of Missing Requirements under 35 U.S.C. 371 in the United States Designated/Elected Office form PCT/DO/EO/905, mailed May 23, 2001, indicating that the oath filed with the above-identified application on April 20, 2001 did not include the signature of the inventors. Applicant hereby submits the Declarations for the above-identified application duly signed by the inventors and a check in the amount of $65 for the surcharge required under 37 C.F.R. §1.492(e). Applicants have also included a copy of the Notification of Missing Requirements dated May

P1017342;1

23, 2001.

Applicants request examination on the merits.

Respectfully submitted,

Date: 7/23/01

Joseph W. Bain
Registration No. 34,290
Mark D. Passler
Registration No. 40,764
Akerman, Senterfitt & Eidson. P.A.
222 Lakeview Avenue, 4th Floor
Post Office Box 3188
West Palm Beach, FL 33402-3188
Telephone: (561) 653-5000

Docket No. 789-47

P1017342;1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

5/a
4/2/02

PRELIMINARY AMENDMENT

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Pages 29 and 30 were unintentionally omitted from the response to the Written Opinion during International Preliminary Examination. Please therefore insert the following text after table 15 and before the claims:

-/- **TABLE 16. 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

a'

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 17. 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.

TABLE 18. 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.

a1 Cont.

TABLE 19: 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 activity	enzymatic

			(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

a1 Cont.

* total quantity of enzymes in hydrolysis media -

IN THE CLAIMS:

1. (Amended) 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

(g) recovering the solid contents.

a2

4. (Amended) A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. (Amended) A method as in claim 1, wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. (Amended) A method as in claim 1 , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

7. (Amended) A method as in claim 1, 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).

8. (Amended) A method as in claim 1, 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. (Amended) A method as in claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

10. (Amended) A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

11. (Amended) A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

Please cancel claim 13 without prejudice.

14. (Amended) A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

Please cancel claim 18 without prejudice

19. (Amended) A method as in claim 15, wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in claim 15, wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

25. (Amended) A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

a5 Cont

27. (Amended) A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

Please cancel claims 28 and 29 without prejudice

30. (Amended) A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) 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 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 of step (b) 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 and esters of acetic acid 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 of step (e);

a6

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Please cancel claims 34 and 35 without prejudice.

36. (New) A method as in claim 1, wherein the ketone solvent is acetone.

37. (New) A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

38. (New) A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

39. (New) A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

40. (New) A method as in claim 15, wherein said marine and aquatic animal material is viscera.

41. (New) A method as in claim 15, wherein the ketone solvent is acetone.

42. (New) A method as in claim 16, wherein said marine and aquatic animal material is viscera.

43. (New) A method as in claim 16, wherein the animal material is selected from krill and *Calanus.*

44. (New) A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

45. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

46. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

47. (New) A method as in claim 1, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

48. (New) A method as in claim 31, wherein the ketone solvent is acetone.

49. (New) A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

50. (New) A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

51. (New) A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

REMARKS

A complete copy of the specification which is to form the basis for the US National Phase of PCT/CA99/00987 is submitted herewith.

Respectfully submitted

Date: 4/20/01

Gregory A. Nelson
Registration No. 30,577
Joseph W. Bain
Registration No. 34,290
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, Suite 400
P.O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: 561-653-5000

Docket No. 789-47

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

ATTACHMENT TO PRELIMINARY AMENDMENT SHOWING MODIFICATIONS

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

In accordance with 37 CFR §1.121, the modifications made to the specification and claims are as follows:

IN THE SPECIFICATION

After table 15 and before the claims (modifications indicated with respect to the PCT application as originally filed)

TABLE [17]16. 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

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EL 740156190 US

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]17. 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.

TABLE [19]18. 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]19: 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 activity	enzymatic

			(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

IN THE CLAIMS

Modifications indicated with respect to the claims existing after International Preliminary Examination

1. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(n) 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;

(o) separating the liquid and solid contents;

(p) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(q) 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;

(r) separating the liquid and solid contents;

(s) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(t) recovering the solid contents.

4. (Amended) A method as in [any of] claim[s] 1 [to 3], wherein steps (b) and (d) are conducted under inert gas atmosphere.

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

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

7. (Amended) A method as in [any of] claim[s] 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).

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

10. (Amended) A method as in claim[s] 1 [to 9], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

11. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

14. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a 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 an 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;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a 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 solvent mixture comprising acetone and ethanol to achieve an 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 solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15 [or 16], wherein the animal material is selected from krill and *Calanus*.

19. (Amended) A method as in [any one of] claim[s] 15 [to18], wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in [any one of] claim[s] 15 [to 19], wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in [any one of] claim[s] 15 [to 20], wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in [any one of] claim[s] 15 [to21], wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in [any one of] claim[s] 15 [to 22], wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein prior to step (a) the marine and aquatic animal material is finely divided[, preferably to an average particle size of 5mm or less].

25. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 [and preferably at least about 90] mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g [and preferably at least about 270 mg/g] of krill extract.

27. (Amended) A method of lipid extraction as in [any one of] claim[s] 1 [to 14], wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

30. (Amended) A method of lipid extraction as in [any one of] claim[s] 15 [to 25], wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) 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 of step (b) 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 of step (e);

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) [The] A lipid fraction [obtained by the method of any one of claims 1 to 25, 27, and 30 to 32] extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Respectfully submitted

Date: 4/20/01

Gregory A. Nelson
Registration No. 30,577
Joseph W. Bain
Registration No. 34,290
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, Suite 400
P.O. Box 3188
West Palm Beach, FL 33402-3188
Telephone: 561-653-5000

Docket No. 789-47

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

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

Date of mailing (day/month/year)
20 June 2000 (20.06.00)

Applicant's or agent's file reference
AML/10857.274

IMPORTANT NOTIFICATION

International application No.
PCT/CA99/00987

International filing date (day/month/year)
21 October 1999 (21.10.99)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

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

State of Nationality:
State of Residence:
Telephone No.: 514 397 7675
Facsimile No.: 514 397 4382
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

DUBUC, Jean, H.
Goudreau Gage Dubuc
The Stock Exchange Tower
Suite 3400, P.O. Box 242
800 Place Victoria
Montreal, Quebec H4Z 1E9
Canada

State of Nationality:
State of Residence:
Telephone No.: 514 397 7675
Facsimile No.: 514 397 4382
Teleprinter No.:

3. Further observations, if necessary:
The new agent's address on the Demand has been considered as a change under Rule 92bis. In case of disagreement, the International Bureau should be notified immediately.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
C. Cupello
Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994) 003362378

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 June 2000 (20.06.00)	
International application No. PCT/CA99/00987	**Applicant's or agent's file reference** AML/10857.274
International filing date (day/month/year) 21 October 1999 (21.10.99)	**Priority date** (day/month/year) 21 October 1998 (21.10.98)
Applicant BEAUDOIN, Adrien et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 May 2000 (16.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer C. Cupello
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992) CA9900987

PATENT COOPERATION TRE. Y

PCT

#6 attachment
09/830146

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
AML/10857.274		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 99/ 00987	21/10/1999	21/10/1998
Applicant		
UNIVERSITE DE SHERBROOKE et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

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

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

[X] Patent family members are listed in annex.

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

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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
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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 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

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 99/00987

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 08198754 A	06-08-1996	NONE	
WO 8401715 A	10-05-1984	AT 42111 T	15-04-1989
		AU 573730 B	23-06-1988
		AU 2204183 A	22-05-1984
		BR 8307587 A	25-09-1984
		CA 1220740 A	21-04-1987
		DK 308684 A	22-06-1984
		EP 0107634 A	02-05-1984
		FI 842555 A,B,	25-06-1984
		JP 1934186 C	26-05-1995
		JP 5262665 A	12-10-1993
		JP 6062994 B	17-08-1994
		JP 5017209 B	08-03-1993
		JP 59501908 T	15-11-1984
		NO 842497 A	21-06-1984
		US 4963491 A	16-10-1990
		ZA 8307888 A	26-09-1984
		SE 8302268 A	23-10-1984
		US 4801451 A	31-01-1989
JP 53112195 A	30-09-1978	NONE	
JP 60035057 A	22-02-1985	JP 1383716 C	09-06-1987
		JP 61052183 B	12-11-1986
JP 04057853 A	25-02-1992	JP 2963152 B	12-10-1999
EP 0732378 A	18-09-1996	JP 8253695 A	01-10-1996
		CA 2171780 A	17-09-1996
		NO 961074 A	17-09-1996
JP 51076467 A	02-07-1976	JP 1000118 C	30-05-1980
		JP 52048191 B	08-12-1977
NO 147365 B	28-05-1982	NO 803582 A,B,	28-05-1982
		DK 515581 A	28-05-1982
JP 59196032 A	07-11-1984	NONE	

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

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The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

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It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

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(71) **Applicant** *(for all designated States except US)*: UNIVERSITE DE SHERBROOKE [CA/CA]; University Boulevard, Sherbrooke, Quebec J1K 2R1 (CA).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: BEAUDOIN, Adrien [CA/CA]; 748, boulevard des Vétérans, Rock Forest, Quebec J1N 1Z7 (CA). MARTIN, Geneviève [CA/CA]; 797, McManamy, Sherbrooke, Quebec J1H 2N1 (CA).

(74) **Agents:** DUBUC, Jean, H. et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, P.O. Box 242, 800 Place Victoria, Montreal, Quebec H4Z 1E9 (CA).

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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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METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

BACKGROUND OF THE INVENTION

This invention relates to the extraction of lipid fractions from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals. More specifically, this invention relates to an improved method of extracting lipid fractions by dehydration with solvents and recovering a solid residue rich in active enzymes.

Lipid fractions obtained from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals have various applications:

Medical applications

Marine and aquatic animal oils and fractions thereof contain various therapeutic agents. For example, it is reported that various marine and aquatic animal oils have anti-inflammatory properties. Marine and aquatic animal oils are also reported as helpful in reducing the incidence of cardiovascular disease. Also, some marine and aquatic animal oils are reported as suppressing the development of certain forms of lupus and renal diseases. As a further example, krill may be used as a source of enzymes for debridement of ulcers and wounds or to facilitate food digestion. Also marine and aquatic oils contain various antioxidants, which may have potential therapeutic properties.

Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. These fatty acids are essential for proper development of the brain and the eye. Marine and aquatic animal oils are also rich in liposoluble vitamins A, D and E and carotenoids.

Cosmetics

Various marine and aquatic animal oils are used for the production of moisturizing creams.

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 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"* 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 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 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.

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)

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*

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 *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

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 (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 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 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 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 (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.

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 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 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 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 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 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 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.

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 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, 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.

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 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).

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

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.

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 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 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 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 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 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

(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.

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 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 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].

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.

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 (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)

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

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 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.

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.

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.

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.

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.

BIBLIOGRAPHY

Bowyer, D.E., Leat, W.M.F., Howard, A.N. and Gresham, G.A. 1962. The determination of the fatty acid composition of serum lipids separated by thin-layer chromatography; and a comparison with column chromatography. BBA. 70: 423-431.

Chandrasekar, B., Troyer, D.A., Venkatraman, J.T. and Fernandes, G. 1996. Tissue specific regulation of transforming growth factor beta by omega-3 lipid-rich krill oil in autoimmune murine lupus. Nutr Res. 16(3): 489-503.

Christensen, M.S., Hoy, C-E. and Redgrave, T.G. 1994. Lymphatic absorption of *n*-3 polyunsaturated fatty acids from marine oils with different intramolecular fatty acid distributions. BBA. 1215: 198-204.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. 1983. Spectrophotometric assay using *o*- Phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci. 66: 1219-1227.

Difco laboratories. 1984. Difco Manual Dehydrated Culture Media and Reagents for Microbiology. 10th ed. Detroit.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem. 226: 497-509.

Goodman Gilman, A., Goodman, L.L. and Gilman, A. 1980. The Pharmacological Basis of Therapeutics. 6th ed. Collier Macmillan Canada ltd, Toronto.

Harwood, H.J. and Geyer, R.P. 1964. Biology Data Book. The Federation of American Societies for Experimental Biology, Washington.

Hellgren, L., Karlstam, B., Mohr, V. and Vincent, J. 1991. Krill enzymes. A new concept for efficient debridement of necrotic ulcers. Int J Dermatol. 30(2): 102-103

Plummer, D.T. 1987. An introduction to practical biochemistry. 3th ed. McGraw-Hill Book Company, London.

Rawn, J.D. 1990. Traité de biochimie. De Boeck-Wesmael, Bruxelles.

Runge, J.A. and Joly, P. 1994. Rapport sur l'état des invertébrés en 1994: 7:0 Zooplancton (Euphausiacés et *Calanus*) de l'Estuaire et du Golfe du Saint-Laurent.

Sargent, J.R. 1997. Fish oils and human diet. Br J Nutr.78 Suppl 1: S5-S13.

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

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

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
7-	acetone [a)]	2,15		
	t-butanol [c)]	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)]	2,28		
	ethyl acetate [b)]	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**	

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.

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.

TABLE 6. EXTRACTION OF FRESH FISH LIPIDS (Mack rel)

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..

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.

TABLE 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**
2-	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
ethyl acetate	22.68	25.77	2.17	2.88	22.98	23.51

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

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 ™.

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

TABLE 16 (continu d). 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.

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.

TABLE 19. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggest d 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

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.

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).

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 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.

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.

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.

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.

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.

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.

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.

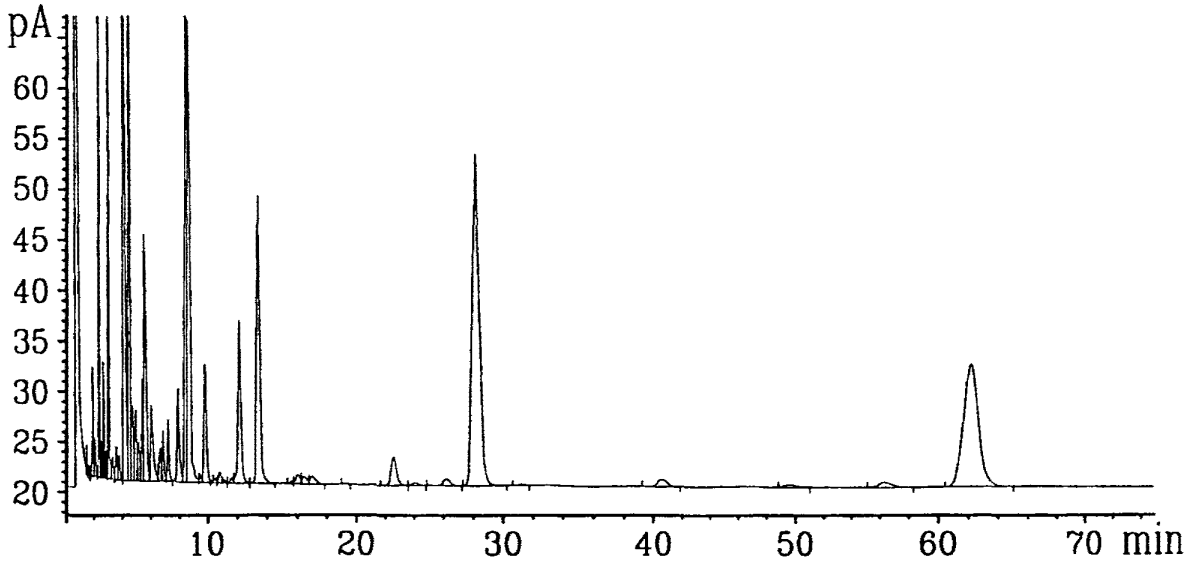

Fig. 1

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	

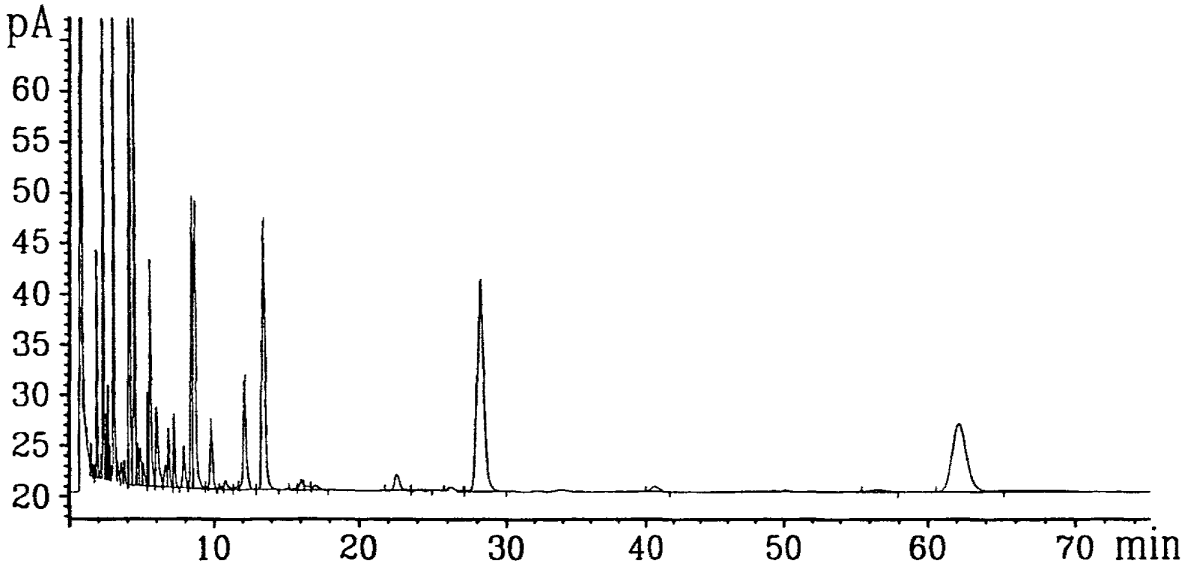

Fig 2

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

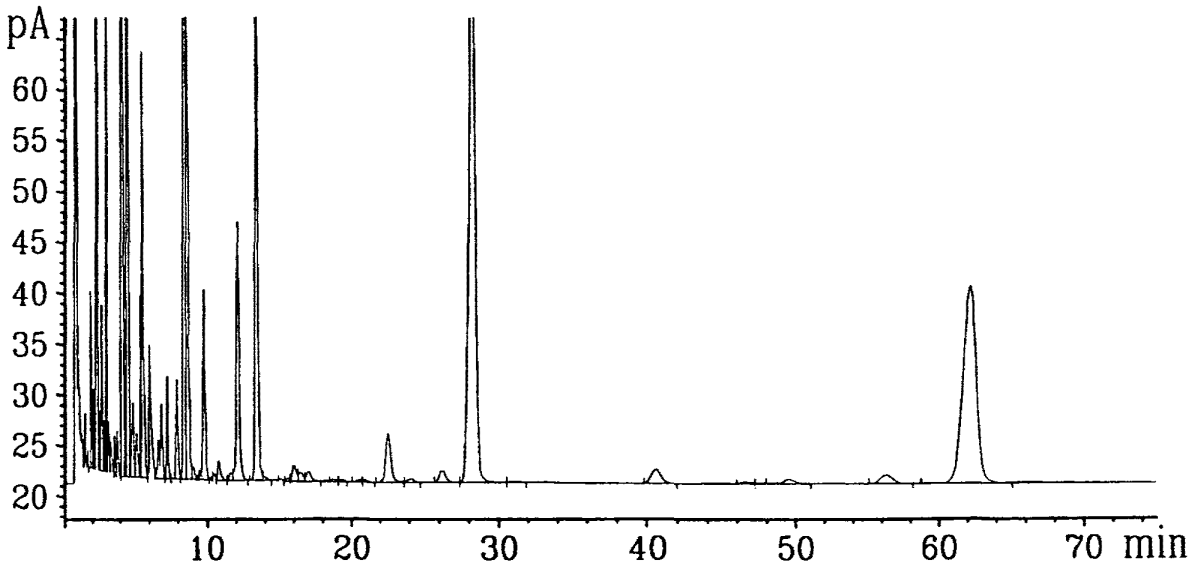

Fig 3

1.216	4.520 - 16:1	13.457
1.262	4.683	13.943
1.454 - 12:0	4.884 - 16:1tr	15.053
1.624	5.030	15.572 - 20:0
1.811	5.111	16.016
1.875	5.420	16.486 - 20:1
2.016	5.561	16.999 - 20:1(cis11)
2.054	6.031	18.762
2.174	6.642	19.303 - 20:2
2.330 - 14:0	6.868	20.474
2.505	7.226	21.027 - 20:3
2.589 - 14:1	7.908 - 18:0	22.575 - 20:4 (6,10,14,18)
2.679	8.444 - 18:1	24.071
2.799	8.639 - 18:1tr	26.215
2.854	9.005	28.333
2.981	9.536	31.180
3.074 - std 15:0	9.788 - 18:2	40.560
3.304	10.267	46.595
3.580	10.481	49.513
3.804	10.807	56.292
4.169 - 16:0	11.626	62.250
4.296	12.140 - 18:3	

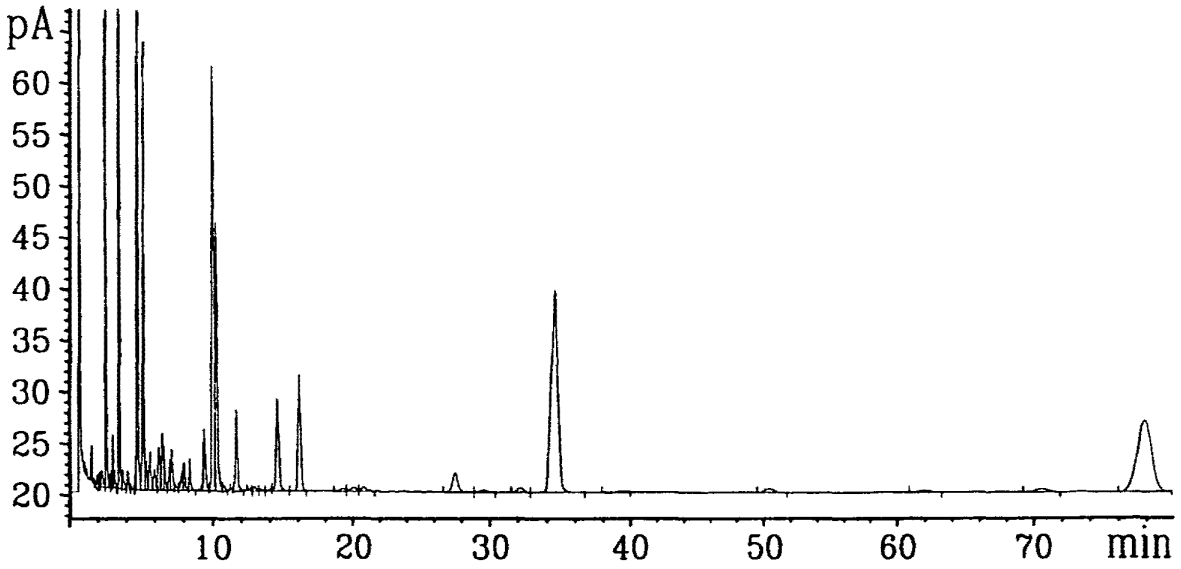

Fig. 4

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	

5/20

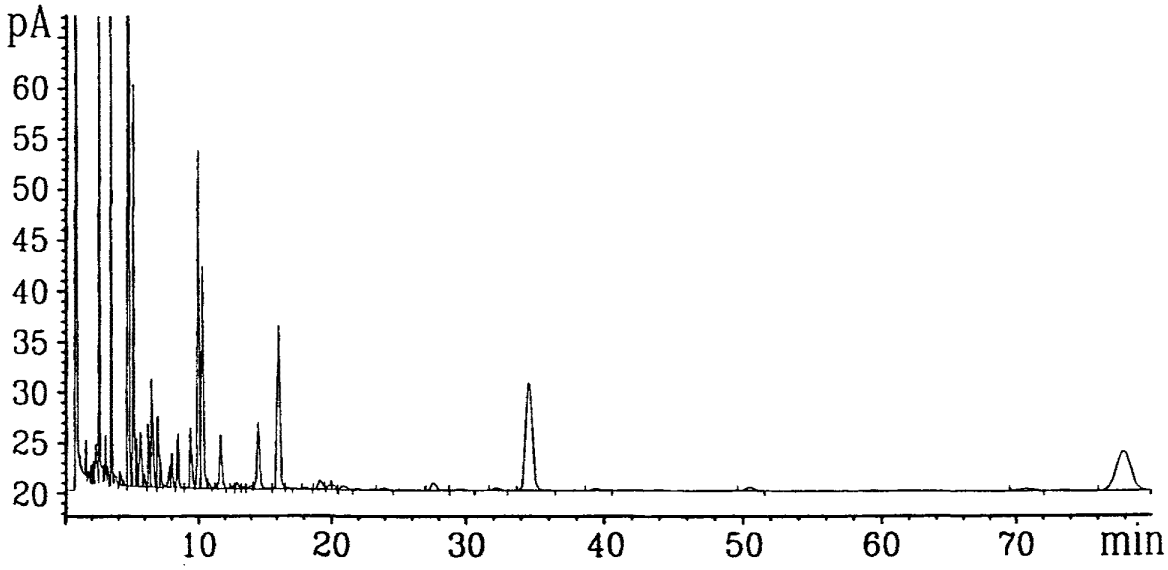

Fig 5

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	

6/20

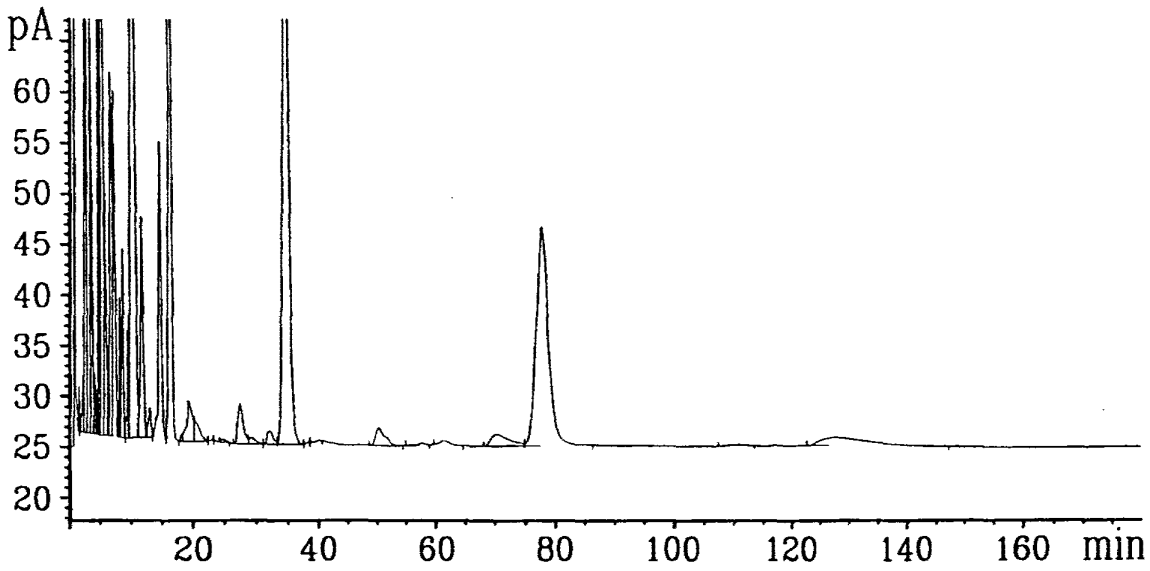

Fig - 6

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	

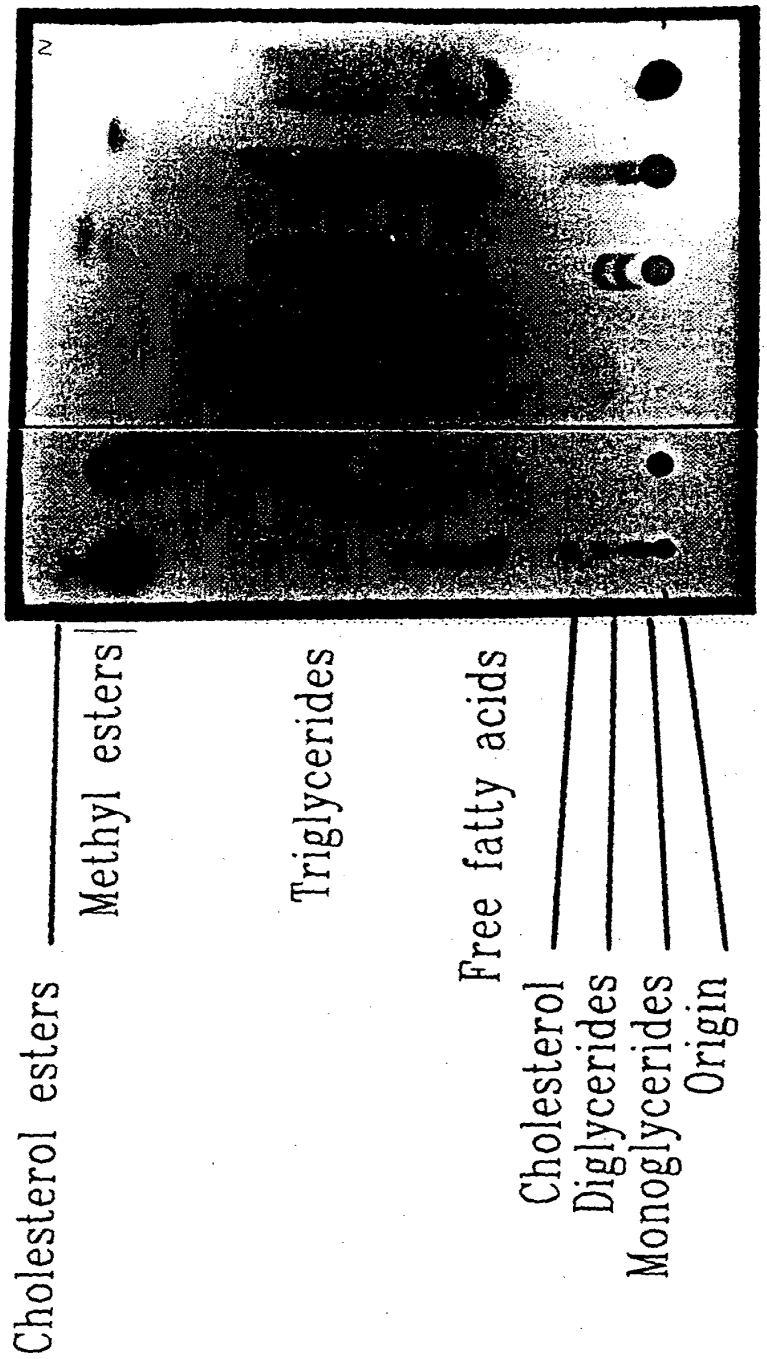
Cholesterol esters
Methyl esters
Triglycerides
Free fatty acids
Cholesterol
Diglycerides
Monoglycerides
Origin

Fig - 7

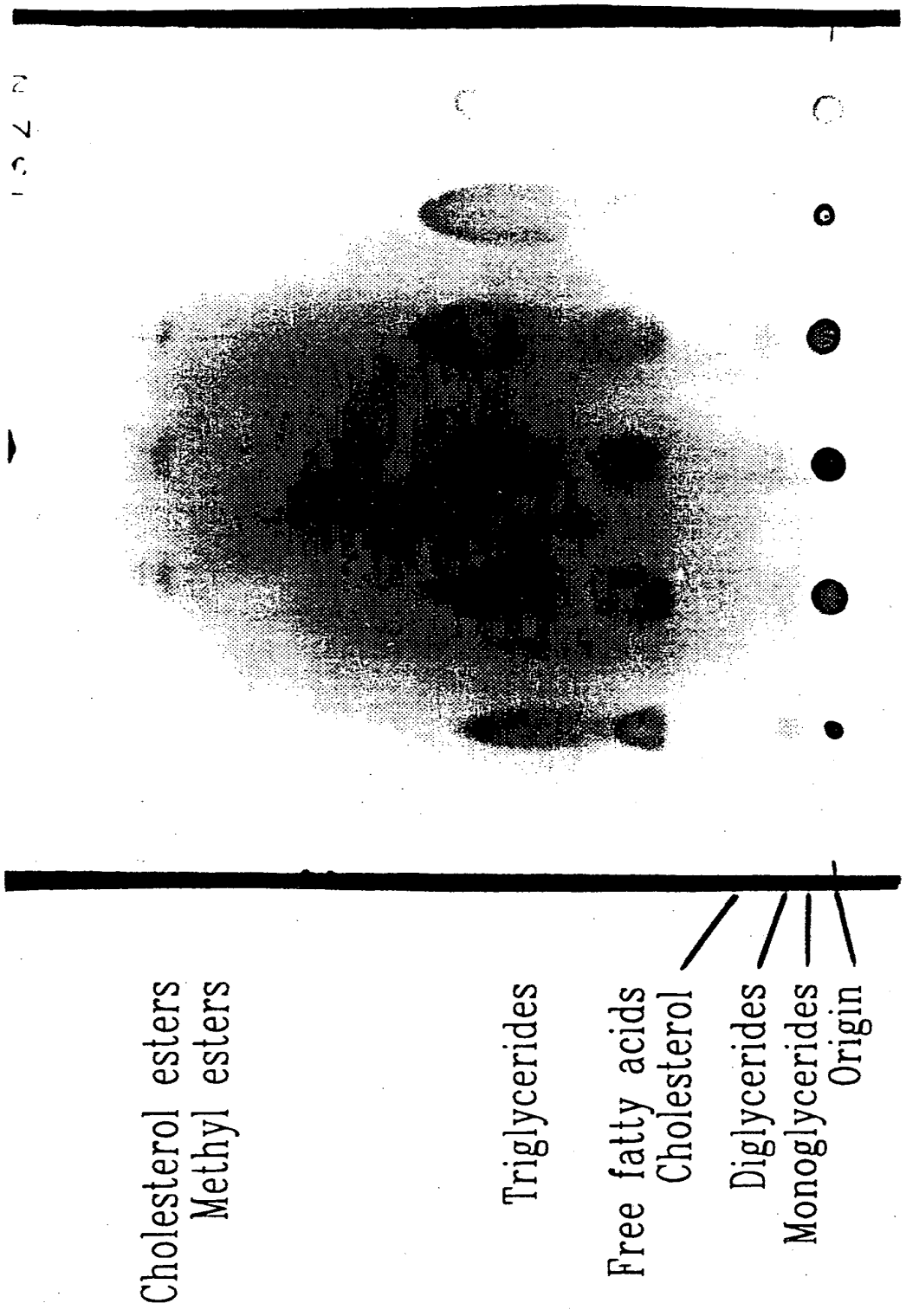
Cholesterol esters
Methyl esters
Triglycerides
Free fatty acids
Cholesterol
Diglycerides
Monoglycerides
Origin

Fig. 8

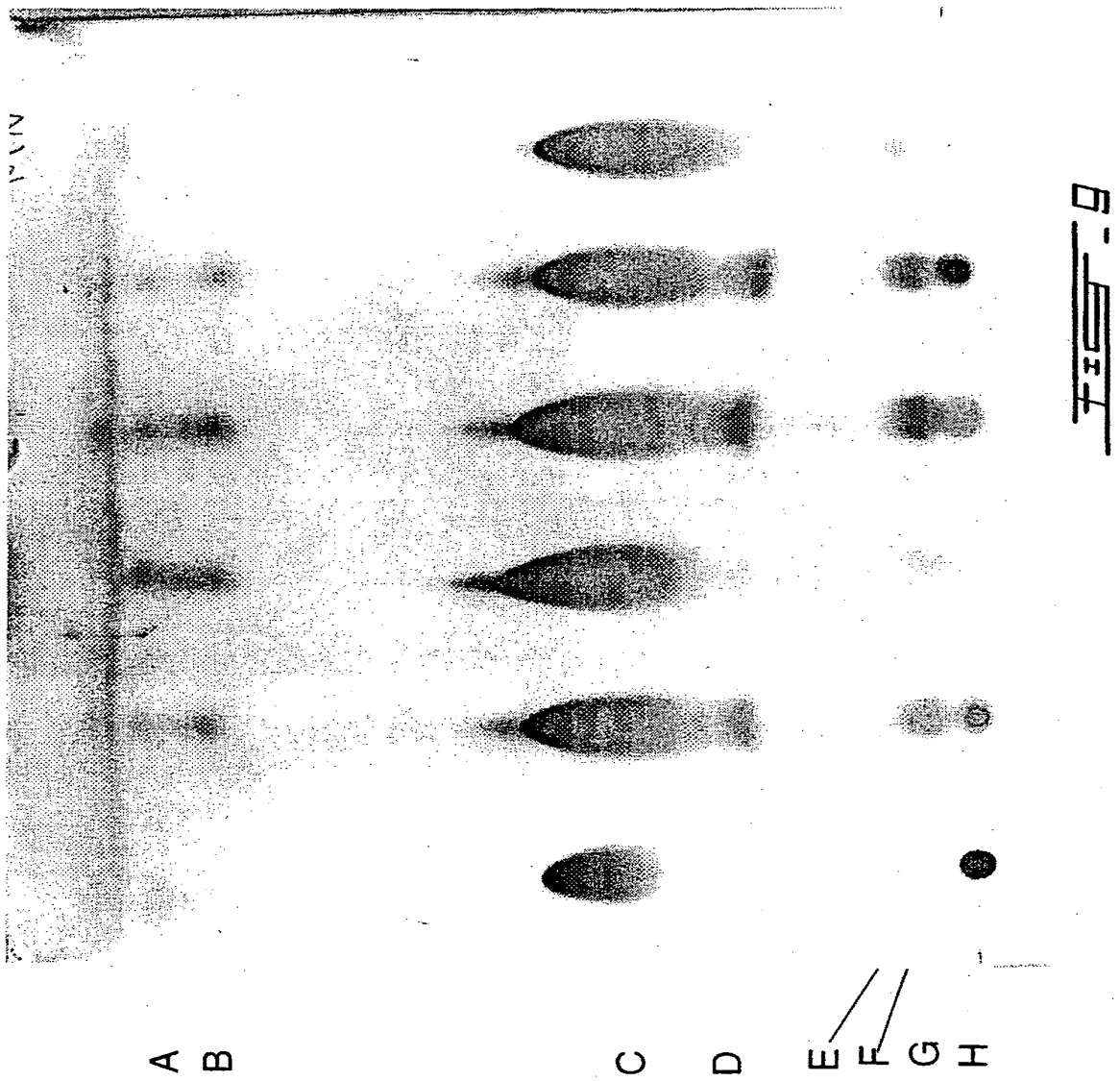

Fig-9

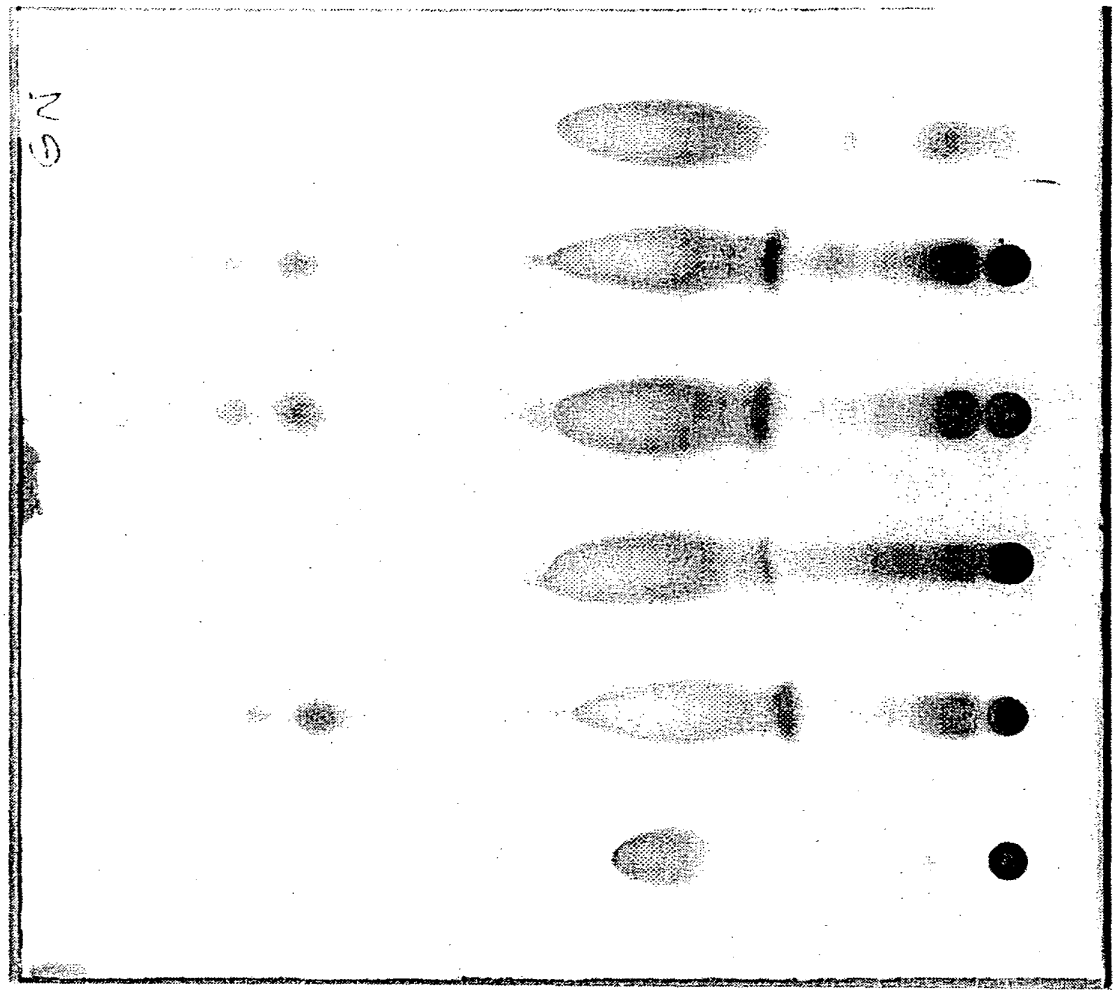

Fig. 10

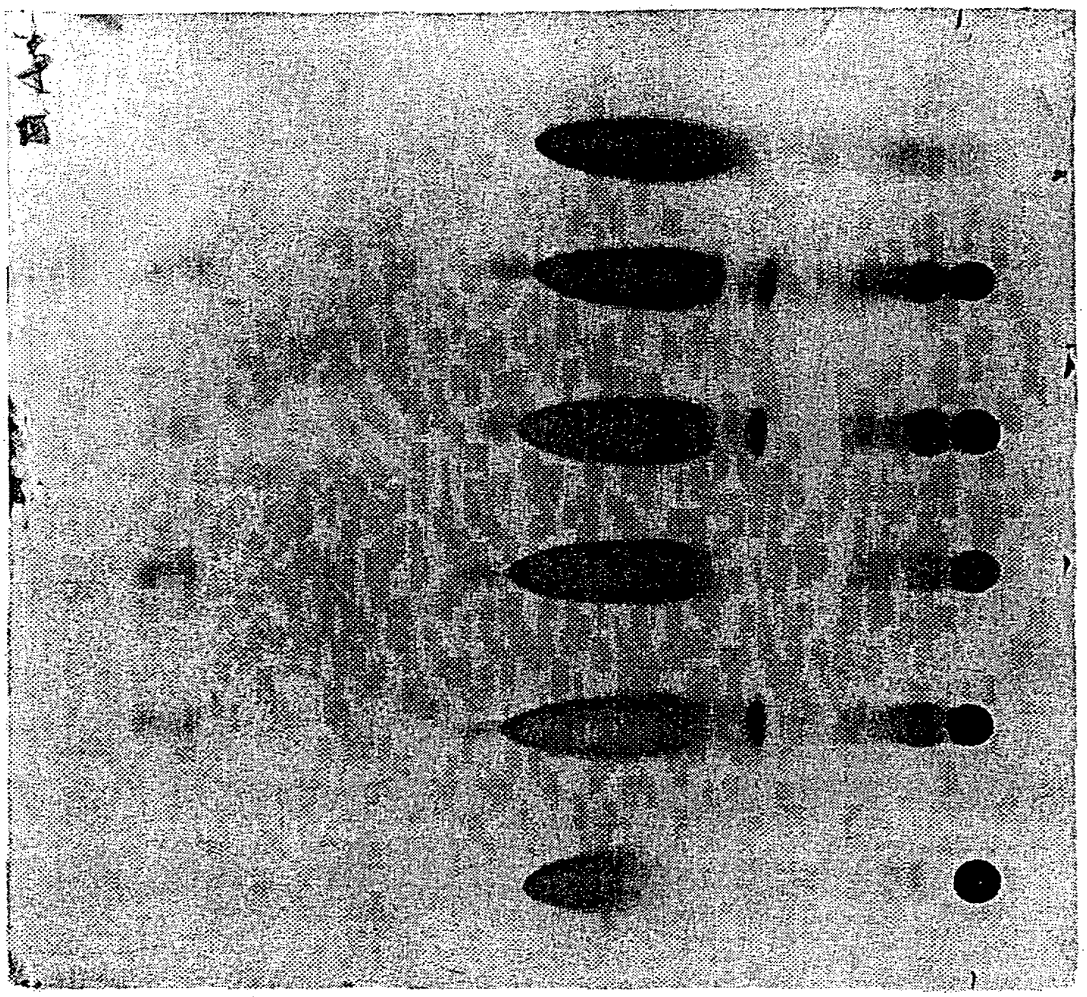

Fig. 11

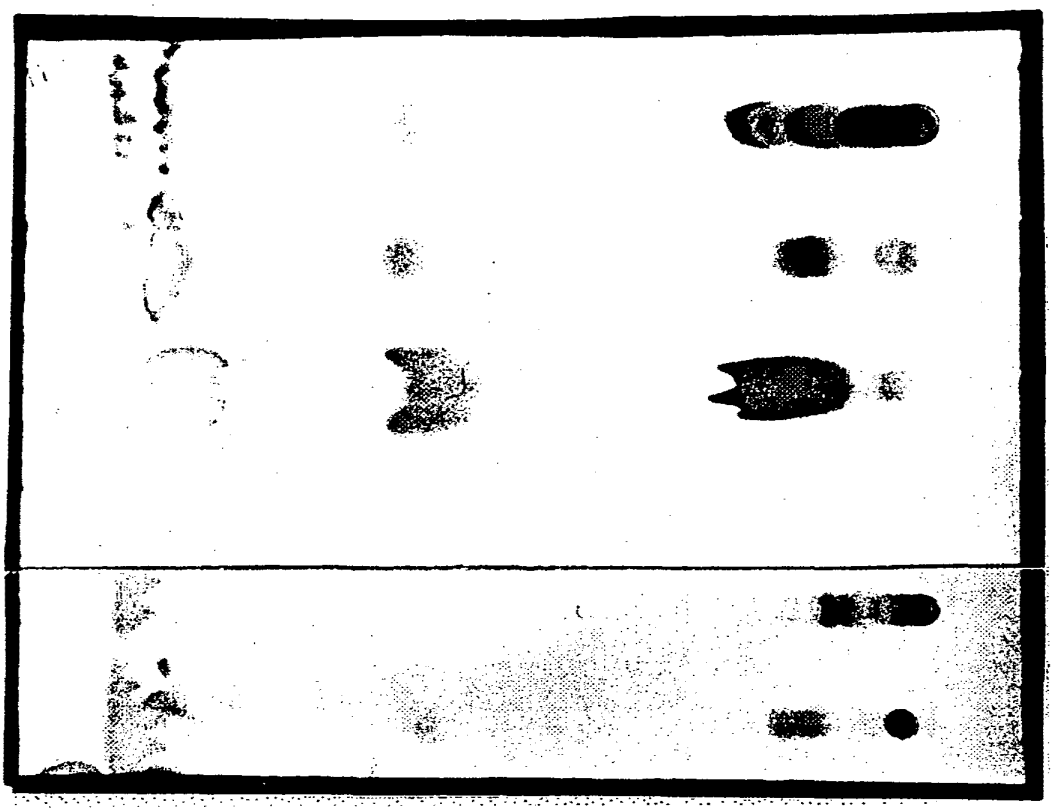
Neutral lipids
Cephalin
Lecithin
Sphingomyelin
Lysolecithin
Origin

Fig. 12

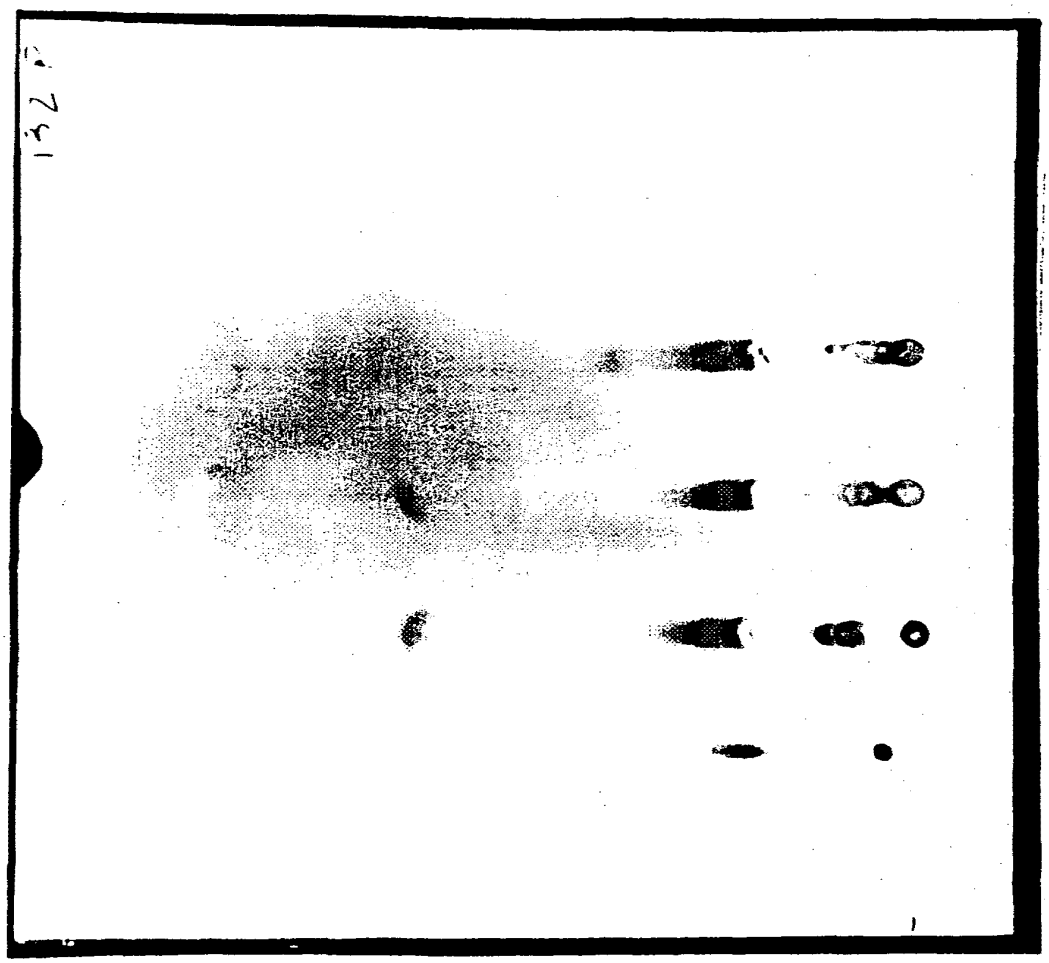
Neutral lipids
Cephalin
Lecithin
Sphingomyelin
Lysolecithin
Origin

FIG. 13

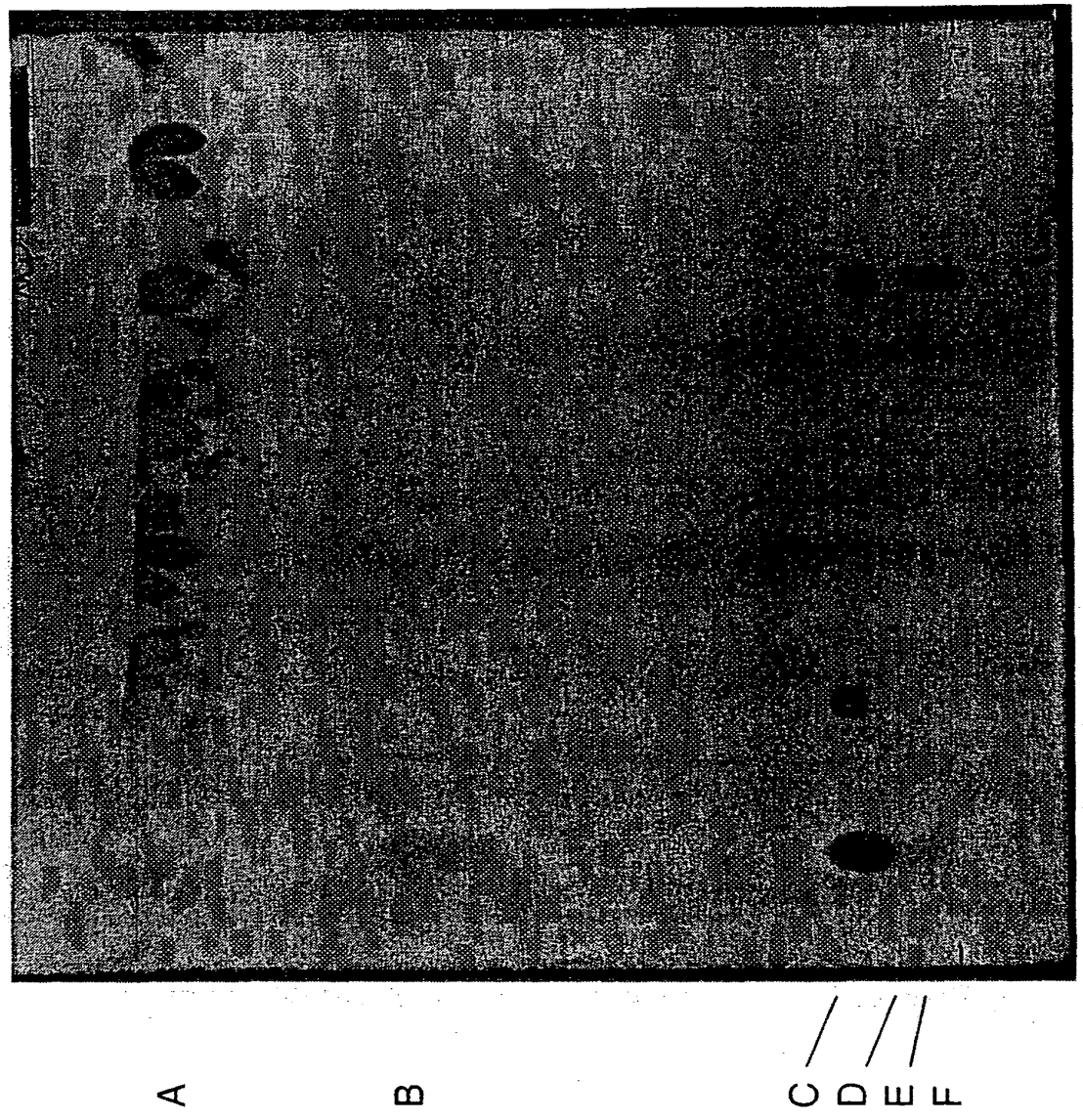

Fig-14

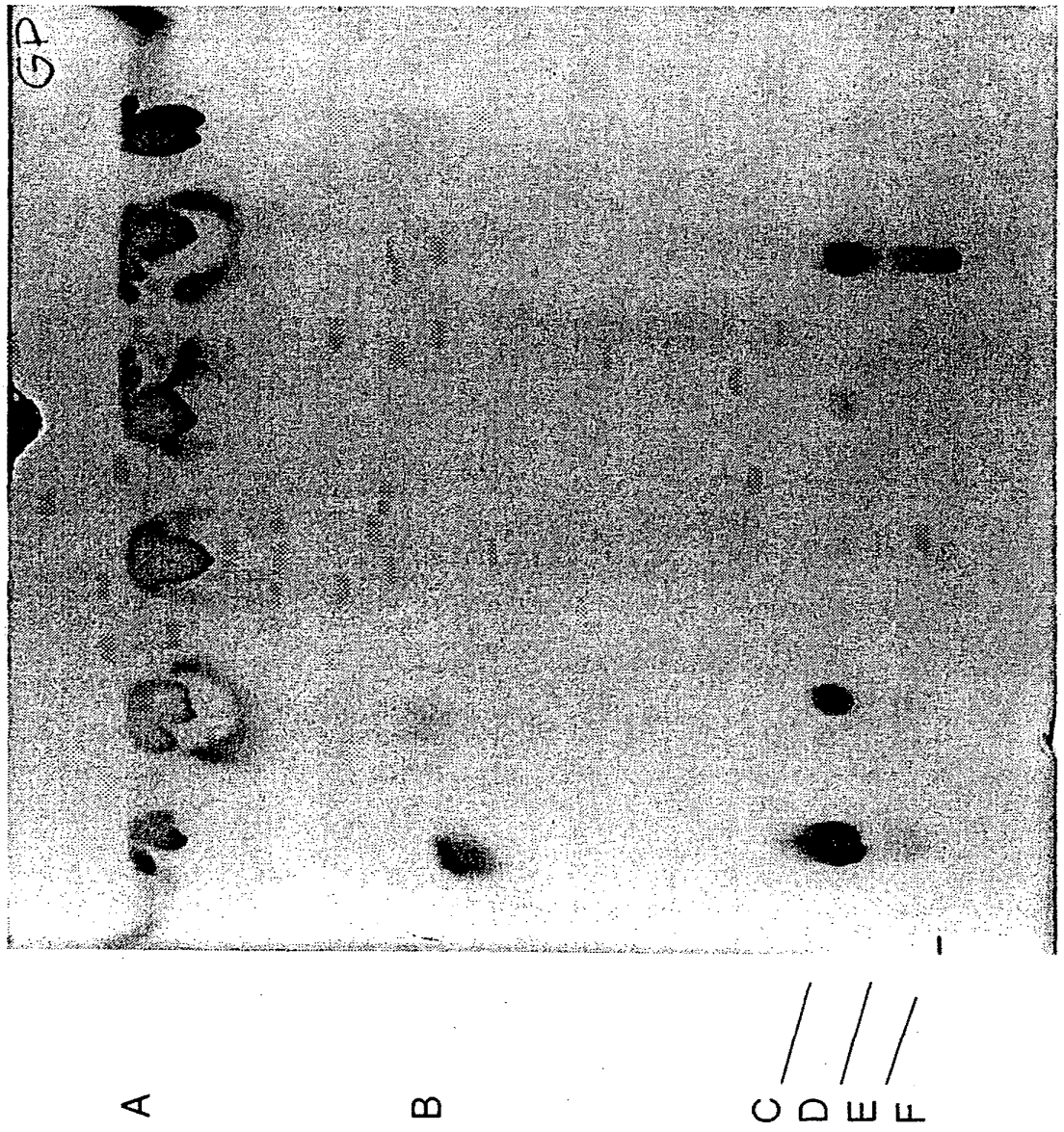

FIG-15

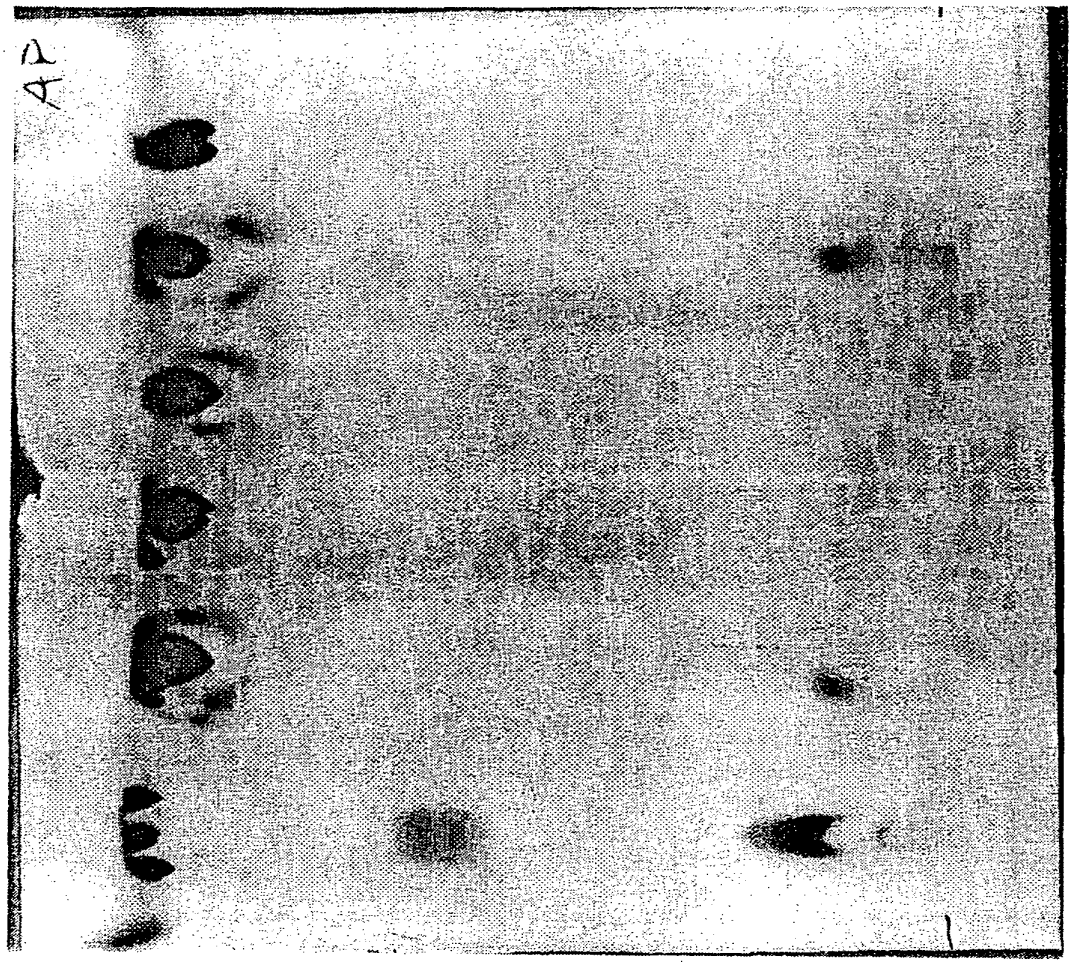

Fig. 18

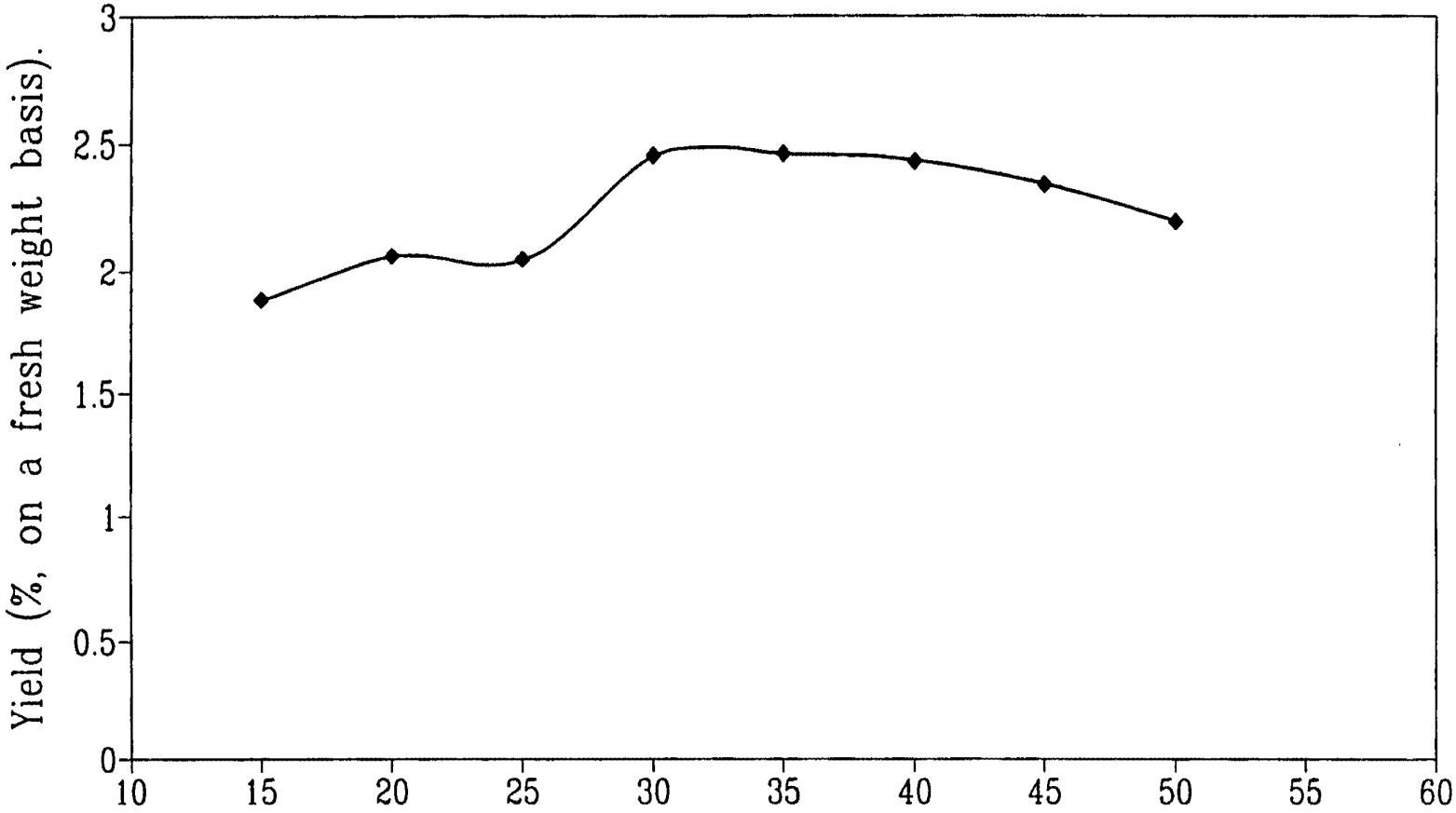

FIG. 17

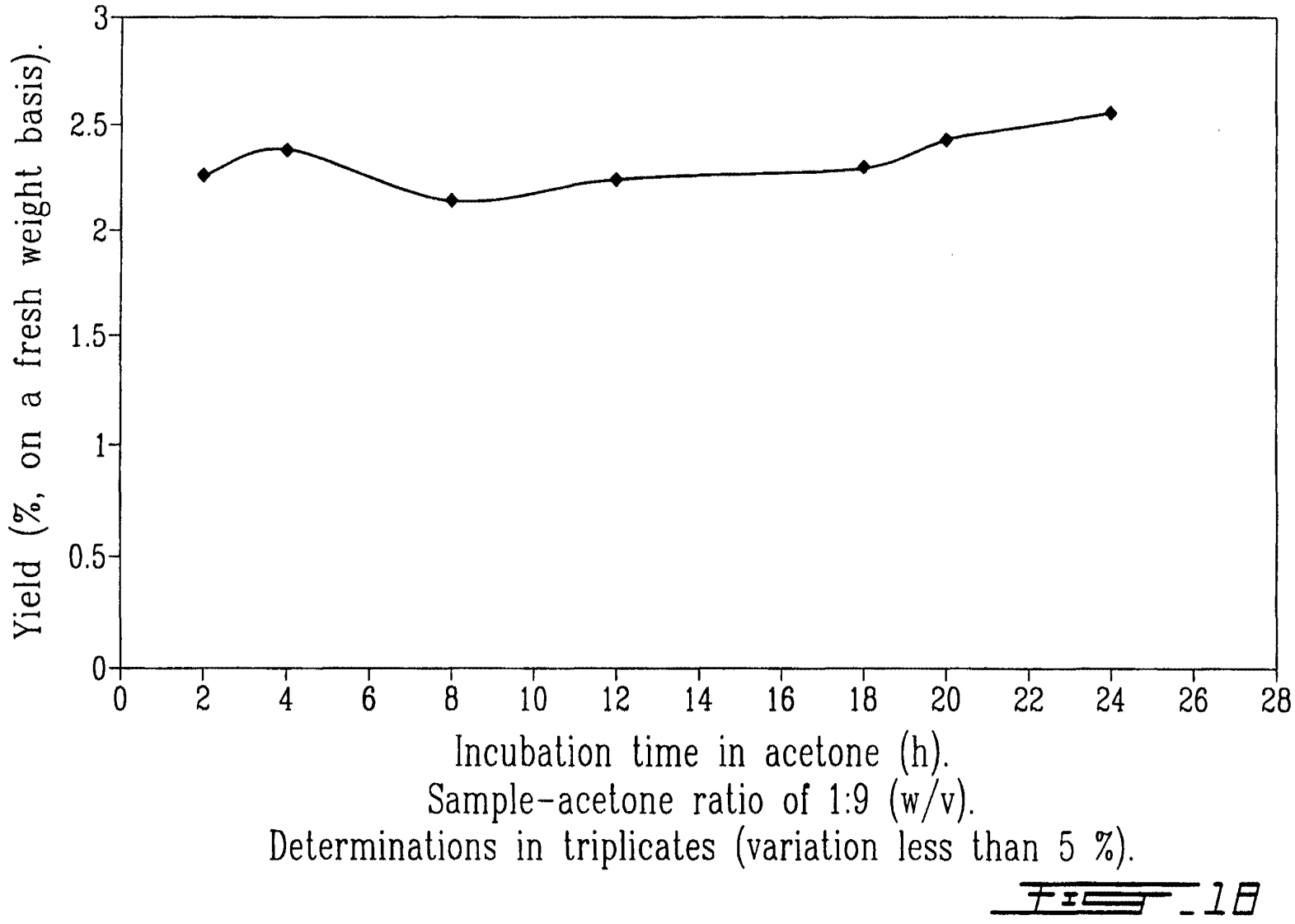

Fig. 18

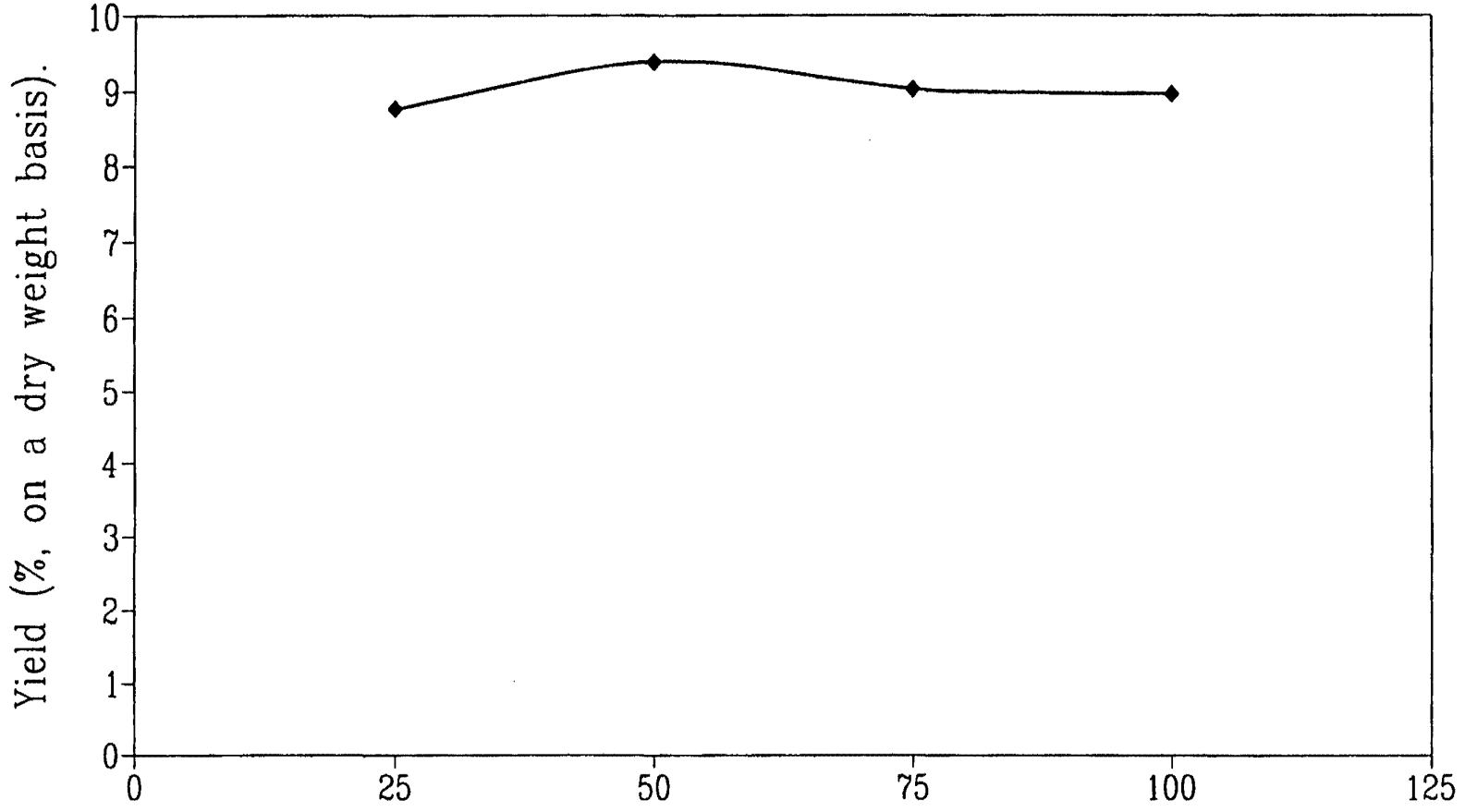

Fig. 19

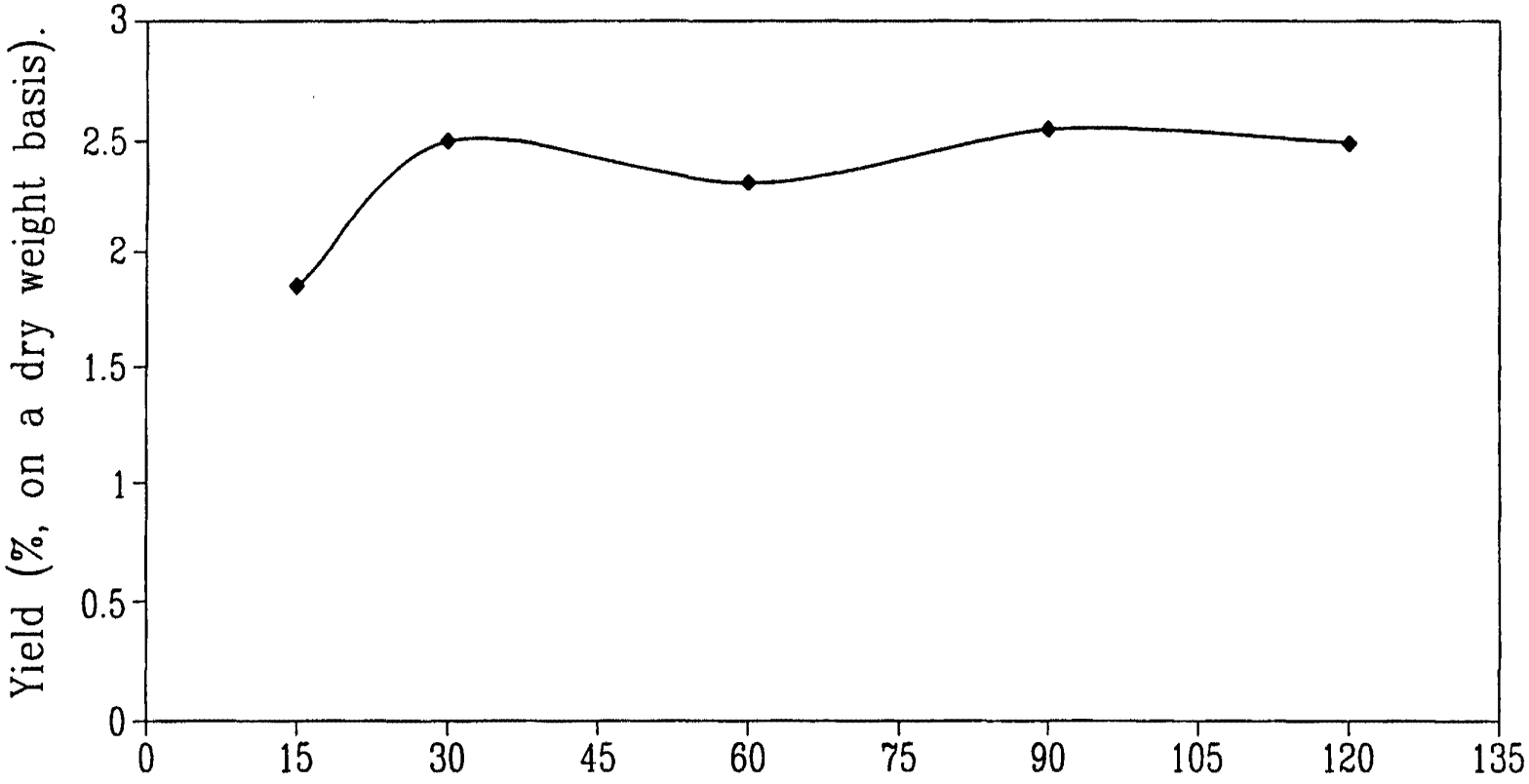
Yield (%, on a dry weight basis).
3
2.5
2
1.5
1
0.5
0
0
15
30
45
60
75
90
105
120
135
Incubation time in ethanol (min).
Sample-ethanol ratio of 1:4 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 20

09/830146

patent application serial no.

Department of Commerce
Patent and Trademark Office
fee record

04/25/2001 LLANDGRA 00000026 09830146

01 FC:971	430.00	OP
02 FC:967	126.00	OP
03 FC:965	80.00	OP

PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2000

Application or Docket Number
09/830146

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	34 minus 20=	* 14
INDEPENDENT CLAIMS	5 minus 3 =	* 2
MULTIPLE DEPENDENT CLAIM PRESENT		☐

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
BASIC FEE	430	OR	BASIC FEE	
X$ 9=	486	OR	X$18=	
X40=	80	OR	X80=	
+135=		OR	+270=	
TOTAL		OR	TOTAL	

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	* 45	Minus	** 45	= 0
Independent	* 6	Minus	*** 6	= 0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X40=		OR	X80=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

(FOR USE WITH FORM PTO-875)

SERIAL NO. 09/830146 | FILING DATE

APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1			1			
2				1		
3				1		
4				1		
5				1		
6				1		
7				1		
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9				1		
10				1		
11				1		
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13				1		
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50						
TOTAL IND.			3			
TOTAL DEP.			26			
TOTAL CLAIMS			31			

	*		*		*	
	IND.	DEP.	IND.	DEP.	IND	DEP.
51						
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100						
TOTAL IND.						
TOTAL DEP.						
TOTAL CLAIMS						

PTO-1360 (3-78) *MAY BE USED FOR ADDITIONAL CLAIMS OR AMENDMENTS U.S. DEPARTMENT of COMMERCE Patent and Trademark Office

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PCT / CA 99/00987

16 NOVEMBER 1999 (16.11.99)

09/830146

REC'D 01 DEC 1999

WIPO PCT

CA 99/987

Bureau canadien des brevets

Certification

Canadian Patent Office

Certification

La présente atteste que les documents ci-joints, dont la liste figure ci-dessous, sont des copies authentiques des documents déposés au Bureau des brevets.

This is to certify that the documents attached hereto and identified below are true copies of the documents on file in the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,,251,265,** on October 21, 1998, by **UNIVERSITÉ DE SHERBROOKE.,** assignee of Adrien Beaudoin and Geneviève Martin, for "Process for Lipid Extraction of Aquatic Animal Tissues Producing a Dehydrated Residue".

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

S. Grégoire

Agent certificateur/Certifying Officer

November 16, 1999

Date

Canada

(CIPO 68)

OPIC CIPO

ABSTRACT OF THE DISCLOSURE

The procedure includes the suspension of freshly collected material in an equal volume of acetone under inert gas atmosphere. Lipids are extracted by successive acetone and ethanol treatments. The procedure produces two lipid fractions and a dry residue enriched in protein and other material insoluble in organic solvents. Recovery of total lipids is comparable or superior to the Folch et al. (1957) procedure. It has been tested with krill, *Calanus* and fish tissues.

TITLE OF THE INVENTION

PROCESS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES PRODUCING A DEHYDRATED RESIDUE

FIELD OF THE INVENTION

The present invention relates to a method for lipid extraction of animal tissues and to the lipid and dry residue fractions obtained therefrom. More particularly, the present invention relates to a lipid extraction method using krill, *Calanus* and fish tissues as starting material.

SUMMARY OF THE INVENTION

Extraction process

Fresh (or frozen) material (*Euphausia pacifica* and other species) is suspended in cold acetone for a given period of time at low temperature (5 C or low r). A ratio of krill-acetone 1:6 (w/v) and an incubation time of 2 h in acetone were found to be optimal. Alternatively the material can be kept in an equal volume of acetone at low temperature for long periods of time (months) under inert atmosphere. The size of the material is an important factor for the penetration of acetone. Indeed, it is preferable to grind material with dimensions superior to 5 mm before getting it in contact with acetone. The suspension is swirled for a short period of time (about 20 min) after acetone addition. After filtration on an organic solvent resistant filter (metal, glass or paper) the residue is washed with two volumes of pure acetone. The combined filtrates are evaporated under reduced pressure. The water residue obtained after evaporation is allowed to separate from the oil phase (fraction I) at low temperature. The solid residue collected on the filter is suspended and extracted with two volumes (original volume of frozen material) of 100% ethanol. The ethanol filtrate is evaporated leaving a second fraction of lipids (identified as fraction II).

Variations of the process

Variable volumes of acetone relative to the levels of sample can be used. It is also applicable to the volume of acetone used to wash and to the volume of ethanol used to extract. Incubation times in solvents may vary. Particle size affect the recovery of lipids and the material could be ground in various sizes of particles, depending on the grinder used. Temperature of the organic solvents and temperature of the sample are not critical parameters, but it is preferable to be as cold as possible.

Methods

To compare the efficiency of the extraction process, a classical technique (Folch et al. 1957) implying chloroform and methanol was applied to krill. This is the standard of reference for the efficiency of the extraction process. Lipid recovery was estimated by suspending lipid fractions in small volumes of their original solvents and measuring by gravimetry small aliquots after evaporation.

To analyze lipid composition, small aliquots of the various extracts were loaded on silica-gel plates 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) including some modifications to the original technique: 1h at 65°C instead of 2h at 80°C, three washes with hexane instead of two and no wash with water.

The dry residue is wetted with ethanol to facilitate a progressive rehydratation of the proteins.

To get rid of traces of organic solvents, lipid fraction I and II are warmed (60°C for fraction I and 70°C for fraction II) for 5 min under inert atmosphere.

Applications

The different fractions (oil, proteins, and others) of aquatic animal biomass xtracted by the current procedure could be used in many fields:

1-Aquaculture

As mentioned in results, fatty acids 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid) are found in high concentrations in krill, *Calanus*, and fish. Farming fish on high quality marine oils rich in docosahexaenoic and eicosapentaenoic (EPA) acids is an efficient means of delivering these essential nutrients in human diets and also efficiently exploiting a strictly limited marine bioresource (Sargent 1997). Krill may be used as food supplement for fish and shrimp (Sargent 1997) because of its capacity to improve growth and survival capacity against diseases (Runge 1994), as pigmentation enhancer for ornamental fish species and as starter diet for marine and fresh water species (Prawn Hatchery Food 1997).

2-Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, the marine oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. 22:6 *n*-3 fatty acid is essential for proper development of the brain and the eye (Sargent 1997).The beneficial effects of *n*-3 polyunsaturated fatty acids in reducing the incidence of cardiovascular disease by lowering plasma triacylglycerol level and altering platelet function towards a more anti-atherogenic state has been reviewed (Christensen 1994). Also, dietary krill oil, like fish oil, can suppress the development of autoimmune murine lupus: EPA substitutes for arachidonic acid, a substrate for cycloxygenase thereby reducing the production of prostaglandins (Chandrasekar 1996). The effects of dietary supplementation with w-3 lipid-rich krill oil includes decreased expression of TGF_{β} in kidneys and of the oncogene--c-*ras* in splenocytes (Chandrasekar 1996). Krill oil has beneficial effects on life span and amelioration of renal disease similar to those previously described in studies with fish oil (Chandrasekar 1996).

3-Animal food

Feeding the animals with omega-3 fatty acids may increase the level of unsaturated fatty acids and decrease cholesterol levels of meat. This property is exploited in the poultry industry to improve the quality of eggs. *Calanus*, in particular, is a full of promise ingredient of domestic animal's food (Runge 1994).

4-Cosmetic industry

Calanus is used for the production of moisturizing creams (Runge 1994).

5-Medical applications

Krill may be used as a source of enzymes for medical application like the debridement of ulcers and wounds (Hellgren 1991) or to facilitate food digestion.

Finally, these marine products are also rich in liposoluble vitamins A, D, E and K and carotenoids that are extracted with lipids. The chitin of krill and *Calanus* could be exploited to protect plants against fungi. Also, marine oils contain unidentified antioxidants which may have potential therapeutic properties.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Results

Note on experimental conditions

The lipid extraction with acetone, then ethanol is practicable under different experimental conditions, as mentioned on page 1 of this document (variation of the process). Moreover, the majority of data shown in this document are from experiments made with sample-acetone ratio of 1:9 (w/v) incubated overnight at 4°C and with sample-ethanol ratio of 1:4 (w/v) incubated 1h at 4°C. In addition, no material has been ground in most experiments. Only later, tests have been made to standardize the method for extraction of lipids with acetone, then ethanol. As shown in Figure 9 and 11, it appears that optimal ratios of sample-solvent are 1:6 (w/v) for acetone and 1:2 (w/v) for ethanol. Figure 10 and Figure 12 show that optimal incubation times are 2 h for the first solvent and 30 min for the second. Grinding has been experimented and it is clear that solvents have a better impact on ground material, as shown in Table 5. Then, experimental conditions are specified for each experiment.

Diagram 1 illustrates the procedure of lipid extraction from frozen krill which is the same used with dry krill and other fresh species as *Calanus*, mackerel, trout and herring.

Interpretation of results

Table 1 shows that higher levels of lipids are extracted by acetone followed by ethanol as compared to the classical procedure of Folch et al. (1957). The same information is found in Table 5 concerning another krill species (*Megayctiphanes norvegica*). Back to Table 1, one can see that the combination of acetone and ethanol as a single step did not improve the extraction process.

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. 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 1984) 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 is lower (3,67 %) than for *E. pacifica* (4,04 %) shown in Table 2. These variations can be attributable to the season of catch.

Table 4 shows the krill composition obtained from experiments 3 and 4 with frozen *E. pacifica* (Table 2). One finds about 83% of water, 4% of lipids and 12% of dry residue.

Table 5 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 wh n the species is large (4,46% versus 3,53 %).

Considerable quantity of lipids were obtained from *Calanus* (Table 6). Some variations in *Calanus* species composition may explain the variations between experiments 1 and 2 (8,22 % and 10,90 % of fresh weight).

When the technique was applied to fish (mackerel) peripheral tissues (mainly muscles) or viscera, an amount of lipids was extracted (Table 7) but it appeared less efficient than the classical method since extractions of the residue with the latter technique allowed us to recover less lipid. Overall, our technique would allow us to exploit parts of fish that are usually wasted after the withdrawal of fillets of the fish or lipid extracts from fishes not used for human consumption. Those fish tissues not used after the transformation of the fish for human consumption could be stored in acetone, then lipids could be extracted with our process. Extraction of lipids from trout and herring were carried out in parallel with the classical method. Results appear in Table 8 and 9. The yield is not significantly different for the viscera whereas with peripheral tissues (muscles) the classical technique is superior (14,93 % versus 6,70 %). Technique using acetone followed by ethanol for trout and herring (and maybe for other species) seems applicable as well as for mackerel. Table 11 shows the suggested procedure for lipid extraction of aquatic animal tissues.

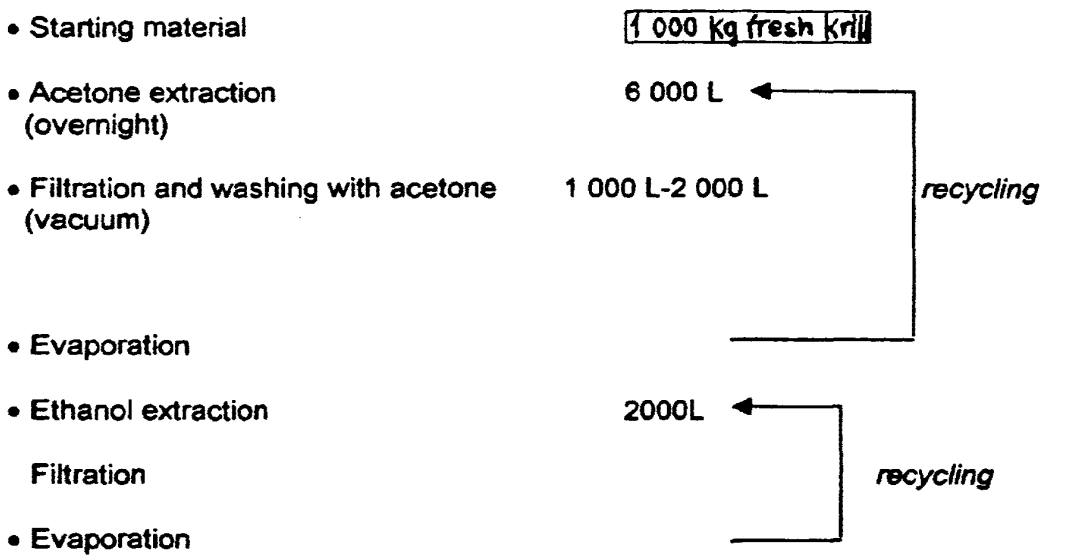
Figures 1 to 4 show chromatograms of fatty acid composition of *E. pacifica* lipids. On each of them, high proportions of 20:5 and 22:6 fatty acids (characteristic of marine oils) are noticeable and represented by two distinct peaks. The concentration of the sample on Figure 4 was lower than the others, so the peaks don't have the same amplitude. With retention times and amounts gave by the chromatograph, identification and compilation of the majority of the fatty acids have been done (see Table 10).

Figures 5 to 8 (TLC) show a higher proportion of neutral lipids as compared to phospholipids in marine oils.

The influence of incubation time on the efficiency of the acetone to extract lipids from *E. pacifica* is illustrated in Figure 9. Extraction is already completed at 2 h. With this time, we proceeded to determine the influence of the sample-acetone ratio (Figure 10). Results show that a ratio of 1:6 (w/v) produce the best yield. The second lipid extraction is carried out with ethanol. The incubation time in this solvent should be at least 30 min as indicated by the results of Figure 11. The volume of ethanol does not appear to be critical since the same yield was obtained with different volumes of ethanol.

One of the inventors, Mr Adrien Beaudoin, has tasted the different lipid fractions. No side effect was observed. The fraction I has the taste of the cod liver oil and the insoluble material tastes like salty shrimps.

DIAGRAM 1. KRILL LIPID EXTRACTION PROCESS



Weight of krill oil : 40 kg (100 lbs)

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

Exp. No.	Technique	Yield (%)	Total (%)
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
			$\bar{x}$=20,49
			σ= 3,95
5-	Chlor : MeOH [c)]		15,60
6-	"		14,90
			$\bar{x}$=15,20
			σ= 0,30
7-	Combined acetone-ethanol [d)]		14,30

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

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

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a)]	2,26	
	ethanol [b)]	2,14	4,40
2-	"	2,25	
		1,13	3,38
3-	"	2,71	
		1,80	4,50 [c)]
4-	"	2,94	
		1,45	4,39 [c)]
5-	"	2,44	
		1,43	3,87
6-	"	2,54	
		1,23	3,77
7-	"	2,58	
		1,46	4,04
8-	"	2,48	
		1,39	3,87
9-	"	2,46	
		1,72	4,18
			$\bar{x}$=4,04
			σ=0,34

Determinations in triplicates (variation < 5 %).

[a)] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

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

[c)] :See Table 4 for total composition.

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

Exp. No.	Technique	Yield (%)	Total (%)
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
			$\bar{x}$=3,67
			σ=0,15

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

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

TABLE 4. FROZEN KRILL COMPOSITION (*E. pacifica*) on a fresh weight basis

Exp. No.	Lipids	Insoluble material	Water
3-	4,50	12,50	83,00
4-	4,39	11,50	84,11
	$\bar{x}$=4,44 σ=0,05	$\bar{x}$=12,00 σ= 0,50	$\bar{x}$=83,55 σ= 0,55

Determinations in triplicates (variation < 5 %).
Experience numbers refer to Table 2.

TABLE 5. INFLUENCE OF GRINDING ON EXTRACTION OF FROZEN KRILL LIPIDS (*M. norvegica*)

Exp. No.	Technique	Krill ground before 1st extraction	Yield (%)	Total (%)
1-	acetone [a)] ethanol [b)]	yes	3,10 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-acetone ratio of 1:6, incubated 2 h at 4°C
[b)] :Extraction made with a sample-ethanol ratio of 1:2, incubated 30 min at 4°C.
[c)] :Folch et al. 1957.

TABLE 6. EXTRACTION OF FROZEN *Calanus* LIPIDS (*Calanus* sp.)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	6,18	
	ethanol [b]	2,04	8,22
2-	"	8,64	
		2,26	10.90
			$\bar{x}$=9,56
			σ=1,34

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.

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

TABLE 7. EXTRACTION OF FRESH FISH.LIPIDS (Mack rel)

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-tissues	"	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-acetone 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-ethanol ratio of 1:4 (w/v), incubated 1h at 4°C.
[c] :Folch et al. 1957.

TABLE 8. 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-acetone ratio of 1:9 (w/v), incubated 1 night at 4°C.
[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.
[c] :Folch et al. 1957.

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TABLE 9. EXTRACTION OF FRESH FISH LIPIDS (H rring)

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-acetone ratio of 1:9 (w/v), incubated 1 night at 4°.
[b] :Extraction made with a sample-ethanol ratio of 1:4 (w/v), incubated 1 h at 4°C.
[c] :Folch et al. 1957.

Table 10: Fatty acid composition *E. pacifica*

Solvent	Saturated	Unsaturated				Unidentified	
		Mono	Di	Poly	H-Poly		
chlo-meth	26,18	22,54	1,91	4,31	26,34	18,72	
acetone	21,4	22,18	1,75	4,67	24,52	25,49	
acetone	19,09	22,11	2,03	4,79	30,24	21,72	
ethanol	45,93	22,96	1,23	2,72	11,11	16,05	(500 µg/mL)
	45,96	22,98	1,24	2,48	11,18	16,15	(200 µg/mL)

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

TABLE 11. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (sugg sted proc dure)

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-ethanol ratio of 1:2 (w/v) pure ethanol 30 min 4°C
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

Bibliography

Bowyer, D.E., Leat, W.M.F., Howard, A.N. and Gresham, G.A. 1962. Th determination of the fatty acid composition of serum lipids separated by thin-layer chromatography; and a comparison with column chromatography. BBA. 70: 423-431

Chandrasekar, B., Troyer, D.A., Venkatraman, J.T. and Fernandes, G. 1996. Tissue specific regulation of transforming growth factor beta by omega-3 lipid-rich krill oil in autoimmune murine lupus. Nutr Res. 16(3): 489-503

Christensen, M.S., Hoy, C-E. and Redgrave, T.G. 1994. Lymphatic absorption of *n*-3 polyunsaturated fatty acids from marine oils with different intramolecular fatty acid distributions. BBA. 1215: 198-204

Difco laboratories. 1984. Difco Manual Dehydrated Culture Media and Reagents for Microbiology. 10th ed. Detroit.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem. 226: 497-509

Goodman Gilman, A., Goodman, L.L. and Gilman, A. 1980. The Pharmacological Basis of Therapeutics. 6th ed. Collier Macmillan Canada ltd, Toronto.

Hellgren, L., Karlstam, B., Mohr, V. and Vincent, J. 1991. Krill enzymes. A new concept for efficient debridement of necrotic ulcers. Int J Dermatol. 30(2): 102-103

Prawn Hatchery Food. 1997. http://www.kk-tech.com/krill.html

Runge, J.A. and Joly, P. 1994. Rapport sur l'état des invertébrés en 1994: 7:0 Zooplancton (Euphausiacés et *Calanus*) de l'Estuaire et du Golfe du Saint-Laurent.

Sargent, J.R. 1997. Fish oils and human diet. Br J Nutr.78 Suppl 1: S5-S13

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Although the present invention has been described herein above by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A method for extracting lipids from an aquatic animal tissue comprising the steps of:

a) suspending said animal aquatic tissue in an organic solvent;

b) extracting lipids by successive organic solvent treatment; and

c) collecting said lipids in a first fraction and an organic insoluble fraction.

2. The method of claim 1, wherein said organic solvent of a) is acetone.

3. The method of claim 1 or 2, wherein said organic solvent of b) is selected from at least one of acetone and alcohol.

4. The method of claim 1, 2 or 3, wherein said organic insoluble fraction comprises a dry residue fraction which is enriched in protein.

5. The method of claim 1, 2, 3 or 4, wherein said aquatic animal tissue is at least one tissue selected from the group consisting of krill tissue, *Calanus* tissue and fish tissue.

6. A lipid extract obtained by the method of claim 2, 3, 4 or 5.

7. A protein rich fraction obtained by the method of claim 4 or 5.

8. A lipid extract having the properties in accordance with the present invention.

CONFIDENTIEL

Injection Date : 98-03-24 20:09:39
Sample Name : 7
Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\ALAIN2.M
Last changed : 98-03-24 19:56:07 by Chantal Beaudoin
(modified after loading)

Méthode corrigée lors de l'installation de la nouvelle colonne 12 septembre 1997. Température du four 170 degré C et purge flow = 150 ml/min. Flux dans la colonne : 4,0 ml/min. Augmentation de la température a 175 degré C et le purge flow est descendu a 140 ml/min, le 13 mars 1998.

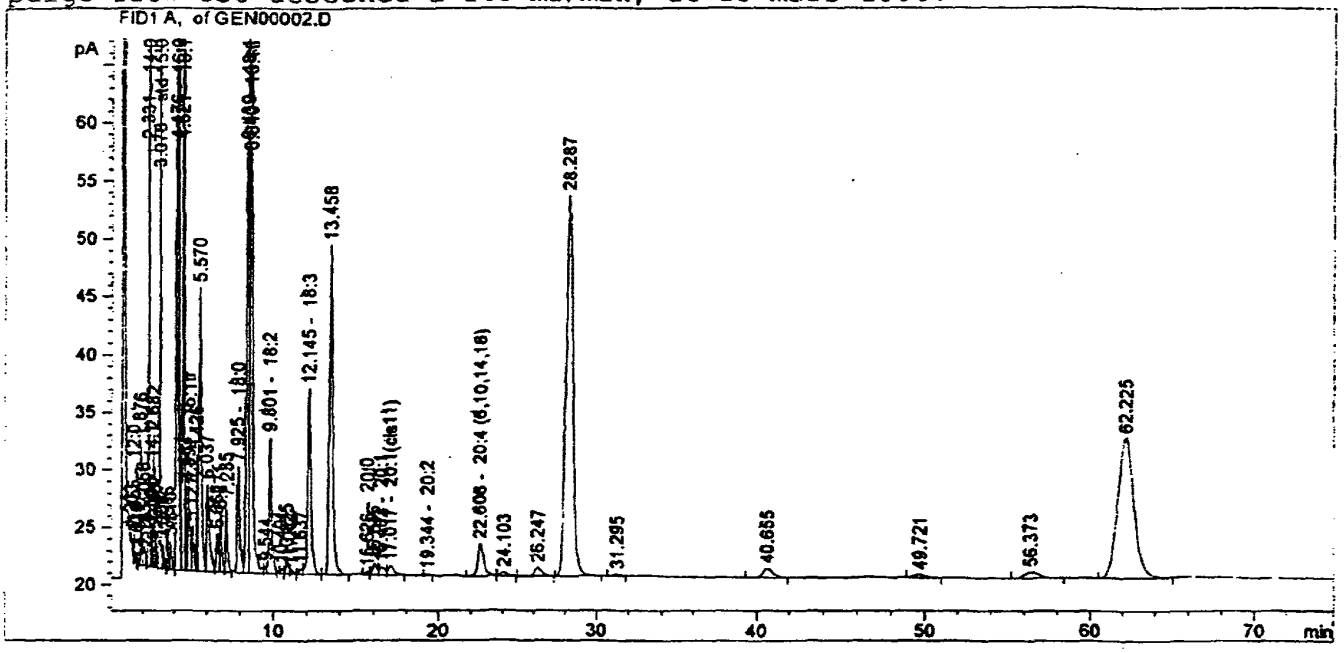

Figure 1: Gas-liquid chromatography of fatty acids from dry krill (chloroform-methanol).

CONFIDENTIEL

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Sample Name : 11 Vial : 1
Acq. Operator : Chantal Beaudoin Inj : 1
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\ALAIN2.M
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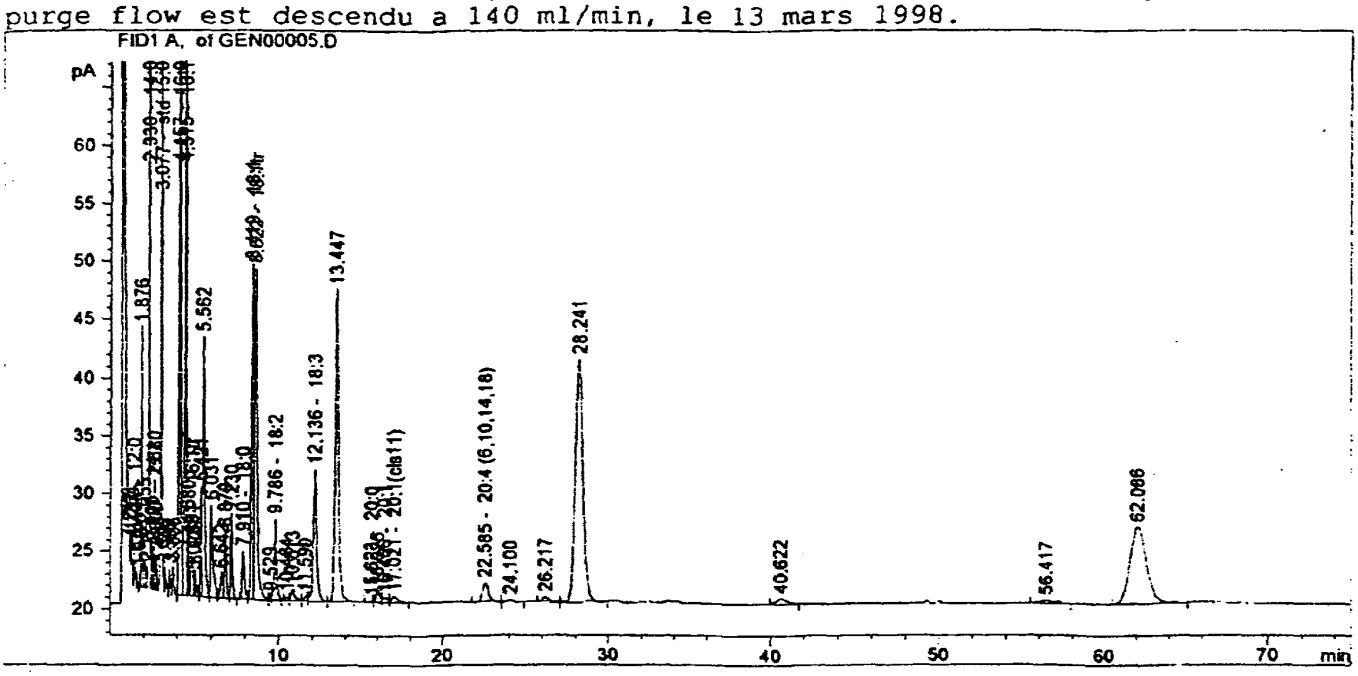

Figure 2: Gas-liquid chromatography of fatty acids from dry krill (acetone).

CONFIDENTIEL

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Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

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Last changed : 98-04-01 18:45:50 by Chantal Beaudoin
(modified after loading)

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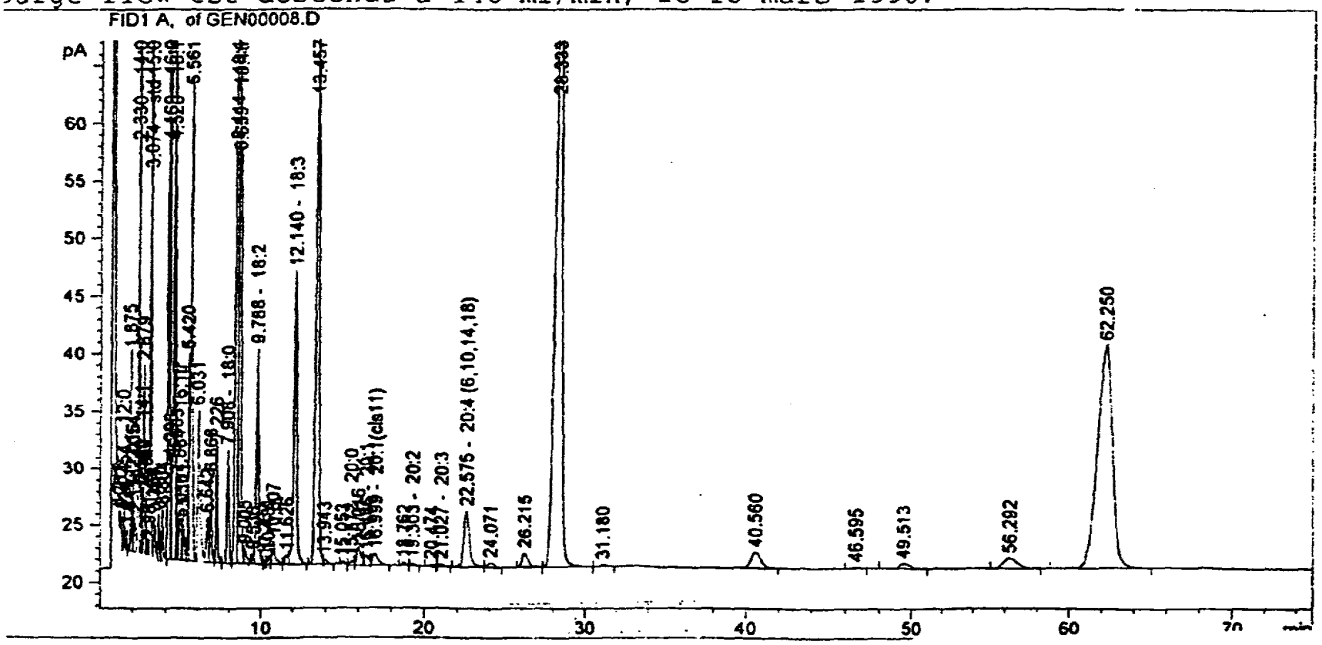

Figure 3: Gas-liquid chromatography of fatty acids from frozen krill (acetone).

CONFIDENTIEL

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Sample Name : 28
Acq. Operator : Chantal Beaudoin

Seq. Line : -
Vial : 1
Inj : 1
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\ALAIN2.M
Last changed : 98-04-02 17:28:39 by Chantal Beaudoin
(modified after loading)

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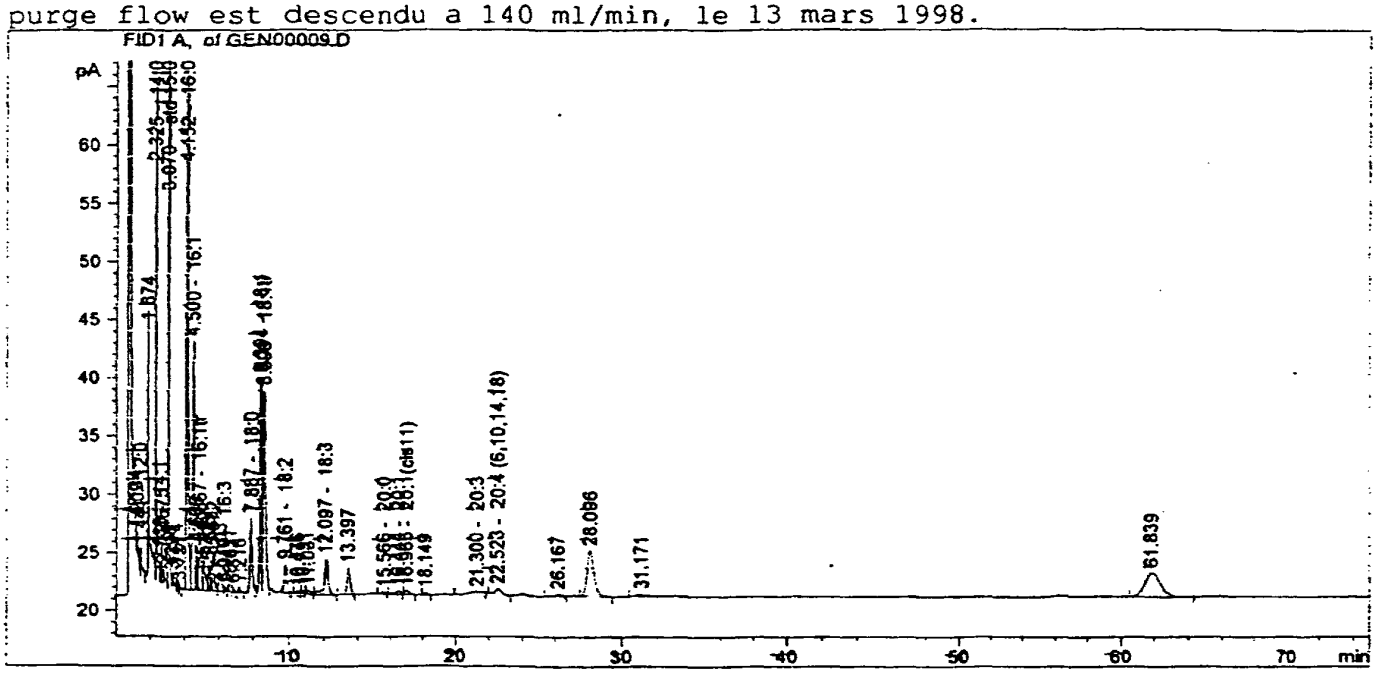

Figure 4: Gas-liquid chromatography of fatty acids from frozen krill (ethanol).

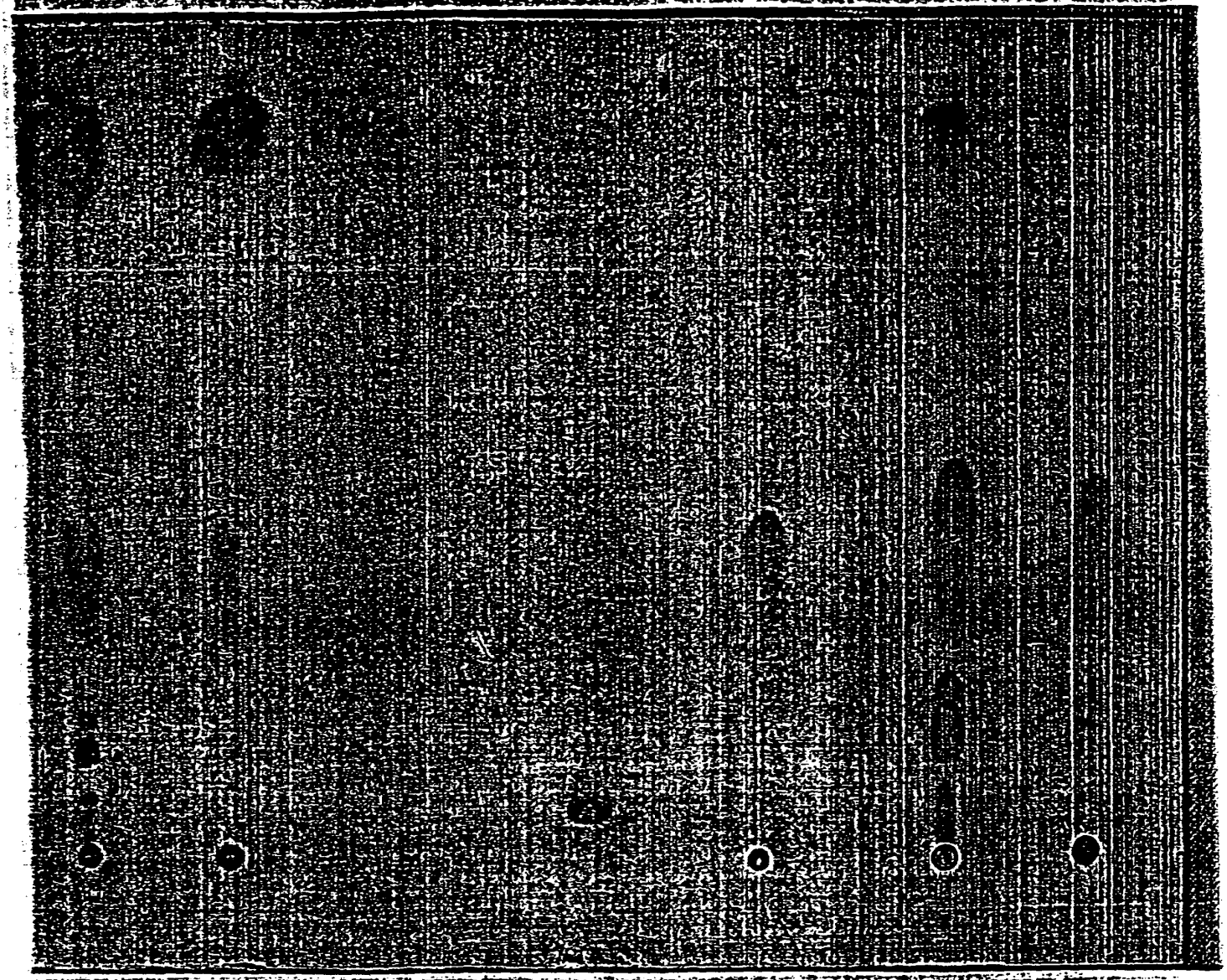

Figure 5: Thin-layer chromatography of neutral lipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), sample of other interest, cholesterol 20mg/mL, egg (acetone), *M. norvegiça* (acetone) and *M. norvegica* (ethanol). Hexane-ethyl ether-acetic acid (90:10:1, v/v).

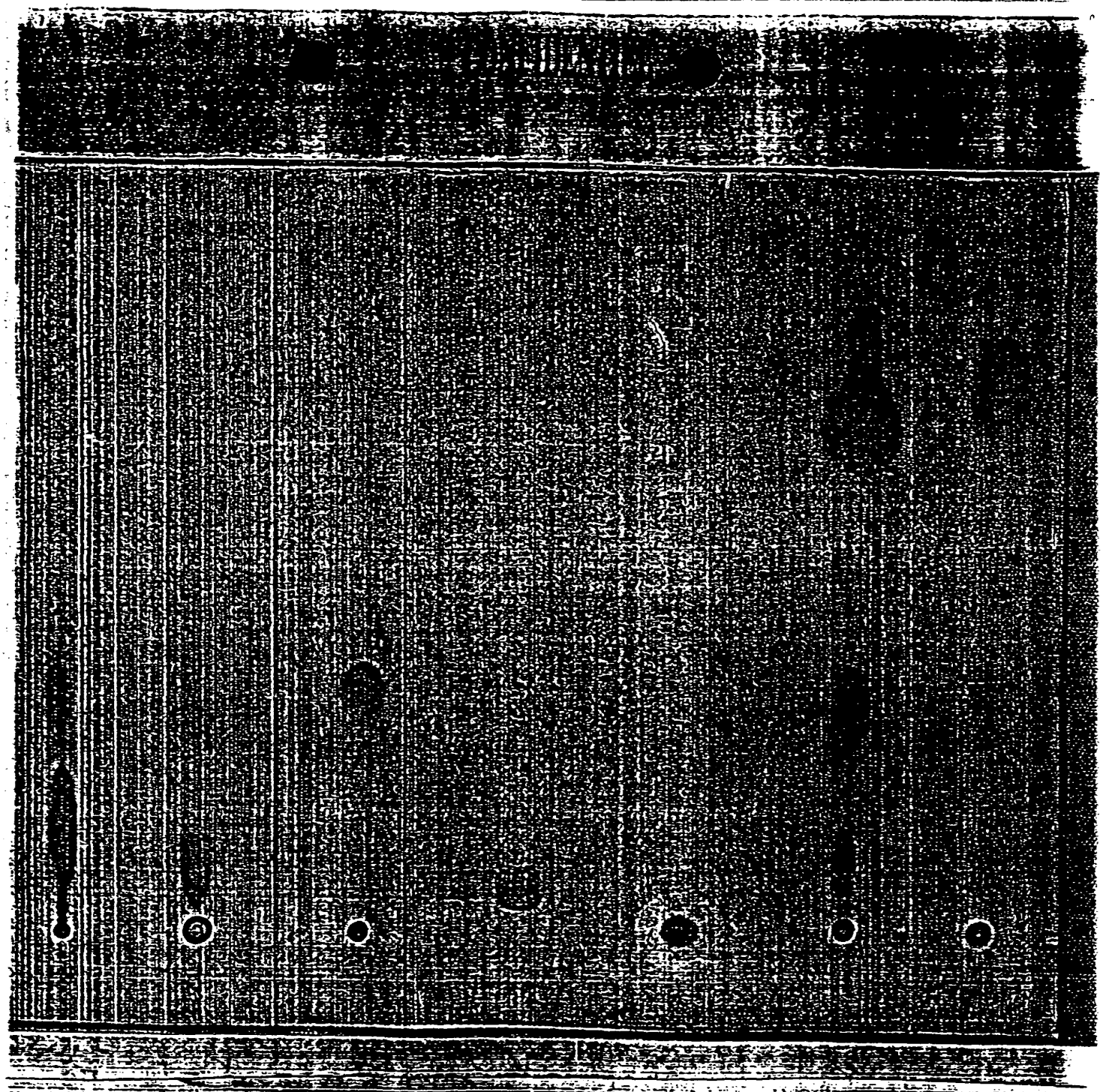

Figure 6: Thin-layer chromatography of neutral lipids of *E. pacifica* (acetone), *E. Pacifica* (ethanol), egg (acetone), cholesterol 20 mg/mL, sample of other interest, *Calanus* sp. (acetone) and *Calanus* sp. (ethanol). Hexane-ethyl ether-acetic acid (90:10:1, v/v).

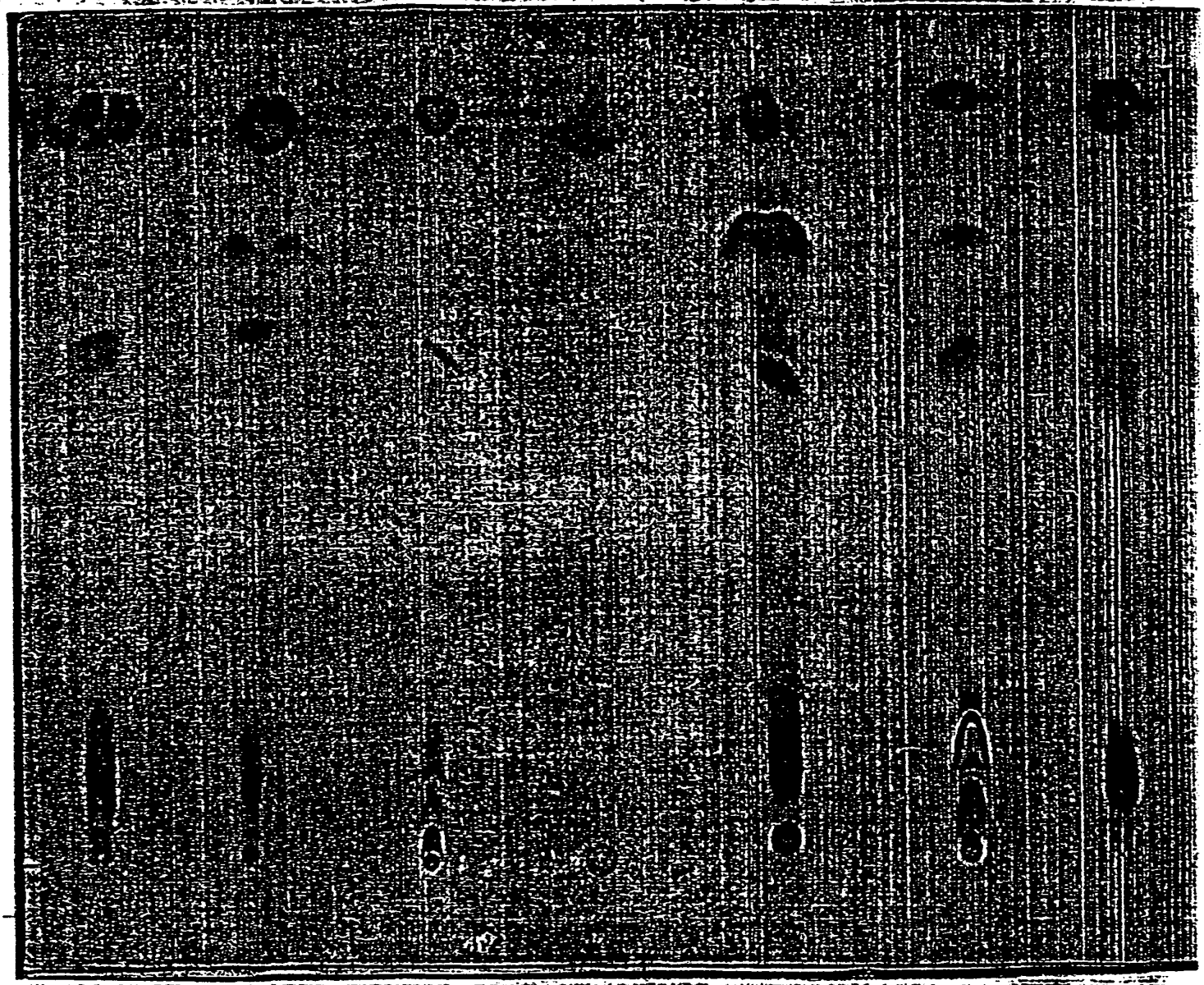

Figure 7: Thin-layer chromatography of phospholipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), cholesterol 20 mg/mL, *M. norvegica* (acetone), *M. norvegica* (ethanol) and egg (acetone). Chloroform-methanol-water (80:25:2, v/v).

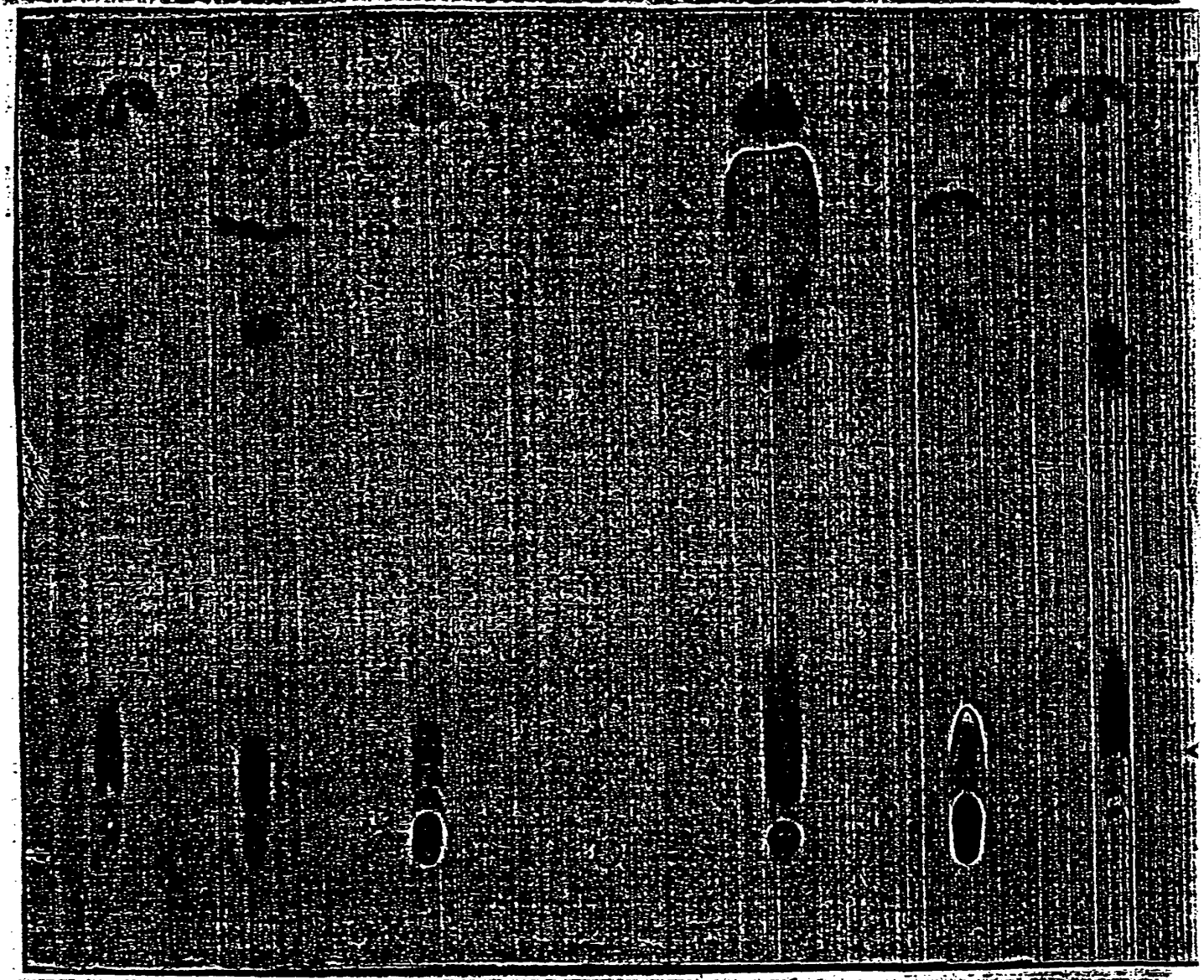

Figure 8: Thin-layer chromatography of phospholipids of *Calanus* sp. (acetone), *Calanus* sp. (ethanol), cholesterol 20 mg/mL, *E. pacifica* (acetone), *E. pacifica* (ethanol) and egg (acetone). Chloroform-metahnol-water (80:25:2), v/v).

FIGURE 9. INFLUENCE OF INCUBATION TIME IN ACETONE ON LIPID EXTRACTION (E. pacifica).

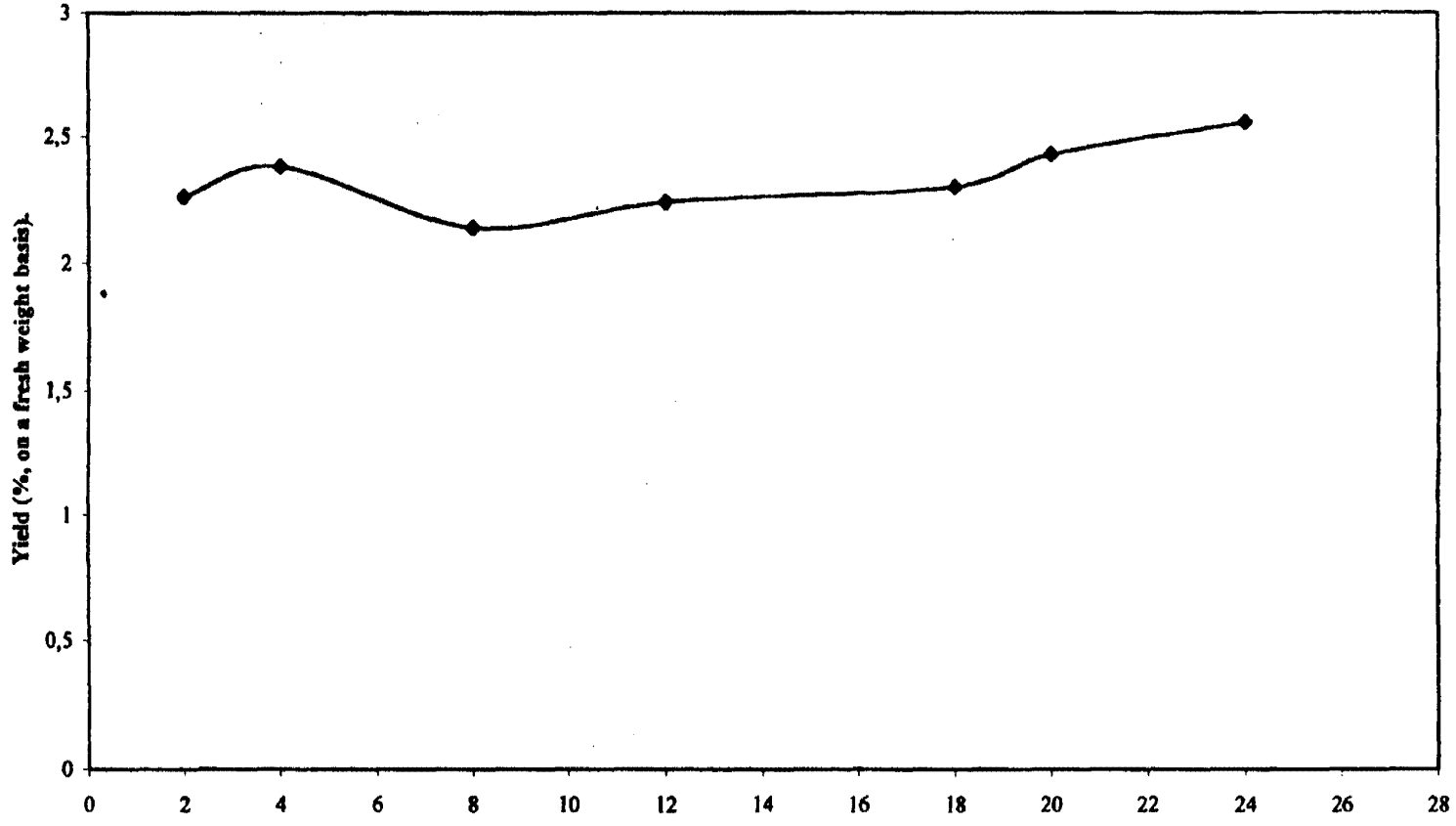

FIGURE 10. INFLUENCE OF THE VOLUME OF ACETONE ON LIPID EXTRACTION (E. pacifica).

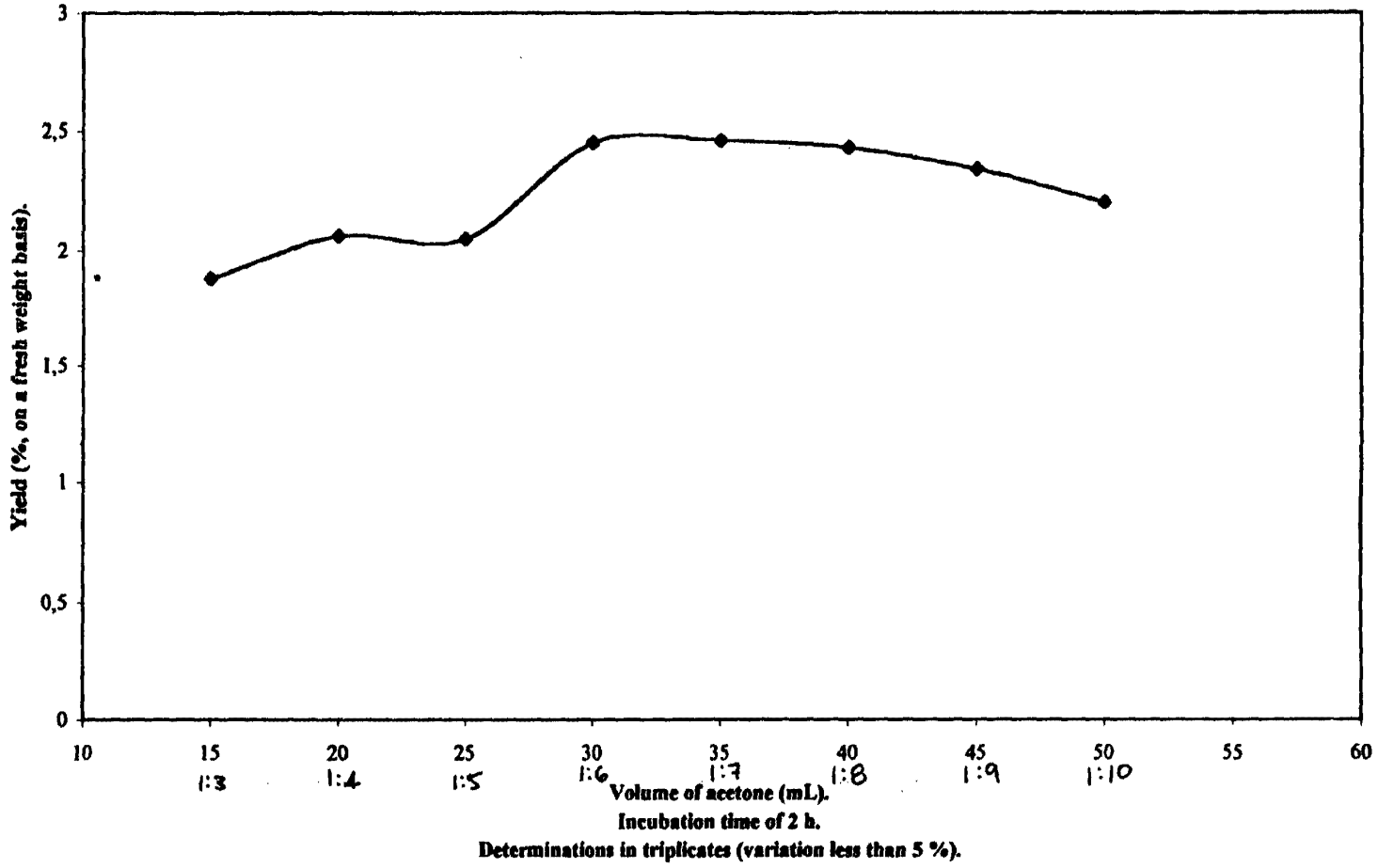

FIGURE 11. INFLUENCE OF INCUBATION TIME IN ETHANOL ON LIPID EXTRACTION (T. raschii).

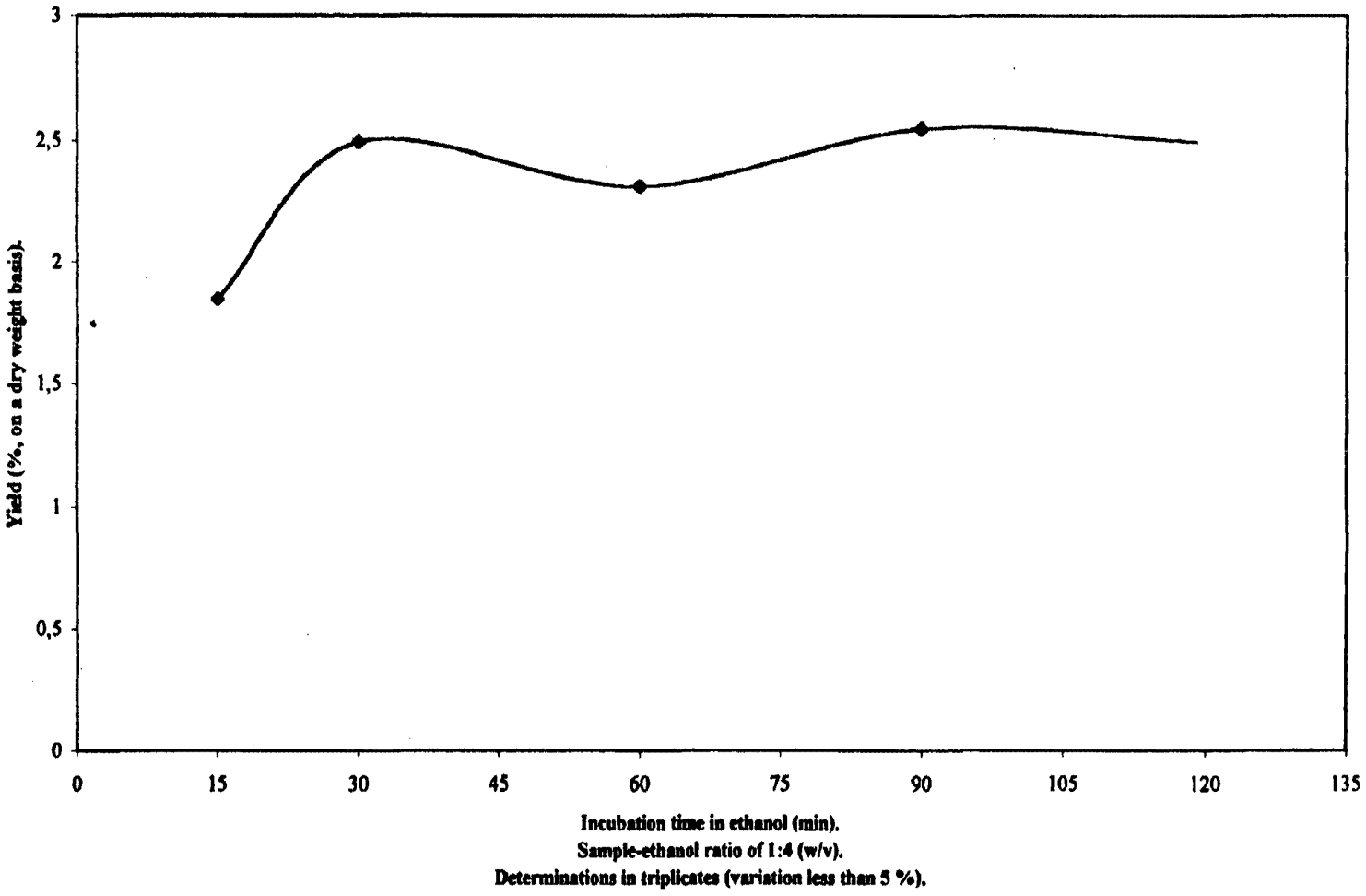

FIGURE 12. INFLUENCE OF THE VOLUME OF ETHANOL ON LIPID EXTRACTION (E. pacifica).