NOAA TECHNICAL MEMORANDUM NMFS SEFC - 241

BIOMEDICAL TEST MATERIALS PROGRAM:

DRUG MASTER FILES FOR BIOMEDICAL TEST MATERIALS, PRODUCED FROM REFINED MENHADEN OIL, AND THEIR PLACEBOS

Sylvia B. Galloway, Ph.D.
Editor

October 1989

U.S. DEPARTMENT OF COMMERCE
Robert A. Mosbacher, Secretary

National Oceanic and Atmospheric Administration
John A. Knauss, Administrator

National Marine Fisheries Service
William W. Fox, Jr., Assistant Administrator for Fisheries

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Appendices 1-5 listed in this document are available as U.S. Department of Commerce, NOAA Technical Memoranda and are referenced as follows:

APPENDIX 1: Joseph, J.D.(ed.) October 1989. NOAA Technical Memorandum NMFS SEFC - 234, "Biomedical Test Materials Program: Production Methods and Safety Manual".

APPENDIX 2: Van Dolah, F.M. and Galloway, S.B.(eds.) November 1988. NOAA Technical Memorandum NMFS SEFC - 211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil".

APPENDIX 3: Fair, P.A. November 1989. NOAA Technical Memorandum NMFS SEFC - 243, "Biomedical Test Materials Program: Distribution Management Manual".

APPENDIX 4: Van Dolah, F.M., Galloway, S.B., and Seaborn, G.T. November 1988. NOAA Technical Memorandum NMFS SEFC - 213, "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules".

APPENDIX 5: Fair, P.A. April 1989. NOAA Technical Memorandum NMFS SEFC - 222, "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil".

Appendix 6 was provided for the specific use of the Food and Drug Administration in its review of this Drug Master File and is not available. The corresponding bibliography is included as Attachment 4.

Copies may be obtained by writing:

National Technical
Information Service
5258 Port Royal Rd.
Springfield, VA 22161

OR

NMFS - SEFC
Charleston Laboratory
P.O. Box 12607
Charleston, SC 29412-0607

DRUG MASTER FILES FOR BIOMEDICAL TEST MATERIALS, PRODUCED FROM REFINED MENHADEN OIL, and THEIR PLACEBOS

Submitted March 1988

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Revised October 1989

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FOREWORD

The information submitted in these Drug Master Files includes chemical composition and processing information pertinent to several assigned Drug Master File Numbers:

* DMF # 7436 - Fish Oils. Information relevant to this DMF # may be found in Sections III, IV, and V. Sections III and IV pertain directly to fish oils, while Section V describes the vegetable oils that are used as placebos with the fish oils.

* DMF # 7779 - n-3 Fatty Acid Ethyl Esters. Information relevant to this DMF # may be found in Sections VI and VII. Section VII describes the ethyl ester preparations of vegetable oils that are used as placebos with the ethyl esters of fish oils as described in Section VI.

General information about the program is provided in Sections I and II. The background and safety information provided in Section VIII pertains to all of the products derived from fish oils and thus it is pertinent to both of the DMF #'s. Additional detailed information on the processing and quality assurance and distribution of the test materials are provided in the Appendices.

The extensive contributions of the scientific staff of the Biomedical Test Materials Program to the preparation of this compilation are gratefully acknowledged. Information on chemical composition, quality assurance, and test material specifications was provided by Fran Van Dolah and Gloria Seaborn and by their staff members Patsy Bell, Jan Gooch, Bennie Haynes, Teresa Icenhour, Richard Johnson, Vijay Koli, Greg Mitchum, Cheryl Sivertsen, and Joe Wilson. Information on process, and production systems and conditions, was provided by Jeanne Joseph and her staff members Tom Brown, Don Duesler, Robert Ernst, Robert Roberts and Joe Wade. Information on storage stability, product forms, packaging, and distribution was provided by Pat Fair and her staff Jim Bonnet. Assistance with hardware and software required for analytical equipment and data handling was expertly provided by Carl Kinerd. Assistance with the many phases of final preparation of a camera ready copy of the document was skillfully provided by Jeannette Smith. In addition I wish to acknowledge the comprehensive reviews provided by Malcolm Hale, Jeanne Joseph and Fran Van Dolah.

SECTION I. OVERVIEW OF THE BIOMEDICAL TEST MATERIALS PROGRAM.

BIOMEDICAL TEST MATERIALS PROGRAM

The Biomedical Test Materials Program (BTM) was formally initiated in 1986 by the signing of a memorandum of understanding (MOU) between the National Oceanic and Atmospheric Administration (NOAA) and the National Institutes of Health (NIH)/ Alcohol, Drug Abuse and Mental Health Administration (ADAMHA) (Attachment 1). Under the MOU it was agreed that the Charleston Laboratory of USDC/NOAA/NMFS would provide a long-term consistent supply of test materials in order to facilitate the evaluation of the role of omega-3 fatty acids in health and disease. An interagency committee, the Fish Oil Test Materials Advisory Committee (FOTMAC), provides the review and approval mechanism for the distribution of quality assured/quality controlled test materials to researchers. The applicants are researchers who are funded by NIH, ADAMHA, and other research organizations.

The unique and important contribution of seafood lipids (oils) to human health really began to unfold with the publication of a series of Danish studies on the low incidence of heart disease in Greenland Eskimos. A number of subsequent studies in this and other countries have led to the hypothesis that increased consumption of seafood or fish oils rich in omega-3 polyunsaturated fatty acids (PUFA) can have direct and positive influence in preventing or ameliorating many degenerative disease processes. At a conference (`Health Effects of Polyunsaturated Fatty Acids in Seafoods', Washington DC, 1985) with leading researchers in these areas, it was concluded that a significant limitation in the research was the lack of adequate supplies of quality assured test materials of consistent composition to explore the many research frontiers identified by the conferees. The BTM Program is designed to respond to this need for reliable test materials which will be available over the period of years necessary to complete the research.

The Charleston Laboratory BTM pilot plant facility was completed in the summer of 1987, including installation and testing of all major production systems and equipment. Installation included a two-stage stainless steel wiped-film deodorizer, a transesterification reaction center, a stainless steel jacketed urea crystallization reactor, a film evaporator and product recovery complex, a two-stage all-glass molecular still, as well as extensively piped nitrogen and chilled water support systems. The installation of a 6 ft stainless steel SCF-CO_2 (supercritical fluid-CO_2) unit was completed in May 1988. The test phase of a `preparative' HPLC (high performance liquid chromatography) unit was completed in June 1988 yielding necessary information required for the selection of a `process' HPLC unit. Installation of the `process' HPLC and an all-glass one-stage solvent stripper was completed in January 1989. All necessary QA/QC procedures are developed, tested and implemented, including a related information management system. The QA/QC Analysis Plan covers both analysis of chemical composition as well as measures of sample oxidation and deterioration. Yearly production rates of vacuum-deodorized menhaden oil, omega-3 PUFA ethyl esters (>85%), EPA ethyl ester (>95%) and DHA ethyl ester (>95%) are: 10,000 kg, 1200 kg, 3.8 kg and 2.3 kg respectively. An adequate quantity of vacuum-deodorized menhaden oil is available to meet the need of all the researchers approved by FOTMAC. On the other hand, the requests for n-3 concentrate and individual n-3 fatty acids exceed the production capacity of the pilot plant. The refined oil and ester

concentrates are available in both bulk and soft gelatin capsules. The corresponding placebo oils are purchased by the researcher; analytical support is provided the researcher via analysis of the purchased oil for tocopherols. Once the tocopherol levels are determined, instructions are provided the researcher as to the proper addition of tocopherols and TBHQ to the placebo oil to balance it with the refined fish oil produced at Charleston. Other available placebos include soft-gel encapsulated vegetable oils and ethyl esters of vegetable oils, as well as bulk ethyl esters of vegetable oils. In the bulk form, the test materials are custom packaged to meet the specific needs of the approved investigator; instructions are provided on handling, storage, and diet preparation to assist the investigator in maintaining the high quality of the test material. Experimental microencapsulation of the test materials was conducted this past year (1989). Storage stability and animal acceptability studies have been conducted and the results will be published in the near future. Purified eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA (>95% each), will be distributed in bulk form only, custom packaged to maintain product integrity, and in limited quantities per researcher. A key feature of the BTM Program is the provision of the same test material (uniform chemical composition and quality) throughout the entire research project, whether it is a few months in duration or several years. The lack of consistently available, high quality, well defined materials has been a significant deterrent to progress in n-3 research.

Research continues in order to refine the methods (SCF-CO_2 and HPLC) that are used to produce the more highly purified individual omega-3 acids (EPA and DHA). The test materials are being used in analytical research, tissue culture experiments, animal and human research studies. All studies involving human subjects are approved pending the `investigative new drug` (IND) approval process through FDA. All IND applicants are required to provide chemical composition, processing, and toxicity information pertinent to the test material being used. This information is customarily provided in a drug master file (DMF).

In response to advice of the FOTMAC, Charleston Laboratory has coordinated the preparation of the materials that were submitted to the FDA in support of the establishment of a DMF for the Test Materials produced at Charleston Laboratory. The file describes the test materials produced by the BTMP, in terms of both chemical composition and process information, and provides background information on the safety of the test materials. The file is referenced by each FOTMAC approved researcher requesting IND approval to use BTMP test materials in human research. In addition to the main body of the DMF, six appendices were prepared: a Production Operations and Safety Manual, a QA/QC Manual, a Packaging and Distribution Management Manual, publications on the storage stability of the encapsulated refined oil and the flavoring of fish oils, and a collection of selected reference materials. Most of these materials are published as NOAA Technical Memoranda (TM). The QA/QC manual is distributed to each approved researcher and provides a complete guide to analytical methods pertaining specifically to fish oil and fish oil fatty acid ethyl esters. The TM describing the 12 month storage stability study of soft gel encapsulated refined fish oil provides detailed information on the composition of the test materials after long-term storage. The TM describing the the evaluation of flavors for masking provides detailed information on the potential for various flavoring agents to mask vacuum deodorized fish oil.

The Production Operations and Safety Manual outlines in detail the daily operations of test materials production. The Packaging and Distribution Management Manual outlines in detail the oversight of the interaction with researchers approved to receive the test materials.

MEMORANDUM OF UNDERSTANDING
SUPPORTING RESEARCH ON SEAFOOD AND FISH OILS IN THE HUMAN DIET
Between the
NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
and the
NATIONAL INSTITUTES OF HEALTH
and the
ALCOHOL, DRUG ABUSE, AND MENTAL HEALTH ADMINISTRATION

I. PURPOSE

The National Marine Fisheries Service (NMFS) of the National Oceanic and Atmospheric Administration (NOAA), United States Department of Commerce, and the National Institutes of Health (NIH) and the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA), agree to cooperate on and provide support to research activities related to the biological mechanisms by which a seafood diet or the ingestion of fish oils may influence health and modulate a number of human disease processes. There is a growing body of research evidence that beneficial effects may be derived from the special components which exist in seafood or fish oils such as the omega-3 polyunsaturated fatty acids (PUFA). Proper evaluation of such components require that specific test materials be made available to researchers in adequate amounts and of consistent quality. This memorandum describes the conditions under which NOAA/NMFS will supply test materials to NIH/ADAMHA-approved research activities including those of independent researchers funded by other sources.

II. REFERENCES AND AUTHORITY

The NOAA enters into this agreement with NIH/ADAMHA under the authority of 16 U.S.C. 742a et seq. (Fish and Wildlife Act of 1956) and 7 U.S.C. 1621 et seq. (the Agricultural Marketing Act of 1946). Applications for the initial NIH/ADAMHA research grants were solicited under an announcement entitled "Biological Mechanisms of Omega-3 Fatty Acids in Health and Disease," issued on December 6, 1985 (NIH Guide for Grants and Contracts, Vol. 14, No. 13). The research objectives, scope, and evaluation process were stated in the announcement. Applicants were advised that regulations (Code of Federal Regulations, Title 42, Part 52 and Title 45, Part 74) and policies that govern the research grant programs of the Public Health Service would prevail. All requests for available test materials, regardless of the source of funding, will be considered under the terms of this memorandum.

III. NOAA/NMFS RESPONSIBILITIES

The NOAA/NMFS will cooperate with NIH/ADAMHA in this program of research under the following conditions:

1. It will produce various types of test materials to be used in NIH/ADAMHA-approved research activities which may include those funded by other sources and considered by NIH/ADAMHA to be sufficiently relevant to this program's objectives to warrant test material support.

2. The forms of test materials expected to be produced will include refined fish oil, concentrates of esters of omega-3 fatty acids, purified omega-3 fatty acids, and deuterated fatty acids. It is expected that during the first year of this program (FY87), the test materials available will consist primarily of refined menhaden oil and concentrates of ethyl esters of omega-3 polyunsaturated fatty acids. The test materials supplied will be accompanied with a written quality assurance to meet agreed upon specifications. Information will also be given on the method(s) of production for each type provided. Test materials will be packaged in containers and in unit amounts appropriate to declared research needs. They will be packaged under conditions that will ensure consistent quality of the delivered product, including instructions on how to store and handle the products. Test materials intended for human use will comply with the requirements of the Food and Drug Administration.

3. Test materials will be supplied only to those researchers approved by a NIH/ADAMHA review committee under the terms of this Memorandum of Understanding (MOU). However, NOAA will assume no liability for its failure to deliver or continue to provide test materials as indicated under this agreement should it be forced for administrative reasons or by technical constraints to reduce or cease its participation as a supplier.

4. The NOAA/NMFS agrees to provide funds for the administrative and technical support of the program at NIH/ADAMHA.

IV. NIH/ADAMHA RESPONSIBILITIES

Proposals for research grants submitted for NIH/ADAMHA consideration will be evaluated as stated in the program announcements and NIH/ADAMHA-supported researchers, including intramural programs, will be given priority in the allocation of available test materials. After being advised by NIH/ADAMHA of the anticipated needs for test materials of all approved research activities, NMFS will determine and report to NIH/ADAMHA the amount and types that can be made available to researchers during the year.

NIH/ADAMHA agrees to:

1. Advise NMFS of both NIH/ADAMHA funded and those studies funded by other sources deemed acceptable to receive test materials, and the quantities and types of test materials requested.

2. Advise NMFS of test material needs to support expected subsequent year grant requirements prior to the beginning of each fiscal year.

3. Advise NMFS as to proper methodologies and procedures necessary for quality assurance and quality control of test materials and to provide technical assistance as required and agreed upon.

V. PERIOD/MODIFICATION

This agreement will become effective upon acceptance by both parties (NOAA and NIH/ADAMHA) and may be modified or terminated by mutual written consent.

VI. OTHER PROVISIONS

Nothing herein is intended to conflict with current NOAA or NIH/ADAMHA directives. If the terms of this agreement are inconsistent with existing directives of either of the agencies, those portions of this agreement which are determined to be inconsistent shall be invalid; the remaining terms and conditions of this agreement not affected by inconsistency shall remain in full force and effect. Changes as are deemed necessary will be accomplished by either an amendment to this agreement or by entering into a new agreement, whichever is deemed expedient to the interest of both parties.

VII. SIGNATURES

Anthony J. Calio
Administrator
National Oceanic and Atmospheric Administration

11/19/86
Date

James B. Wyngaarden
James B. Wyngaarden, M.D.
Director
National Institutes of Health

12.15.86
Date

for Donald Ian Macdonald, M.D.
Administrator
Alcohol, Drug Abuse, and Mental Health Administration

12/29/86
Date

SECTION II. PROCESS FOR TEST MATERIALS DISTRIBUTION.

DISTRIBUTION PROCESS FOR BIOMEDICAL TEST MATERIALS

The system for distribution of the Biomedical Test Materials (BTM) produced by the Program consists of processing by the Fish Oil Test Material Distribution Committee (FOTMDC), a subcommittee of an interagency committee (FOTMAC) administered in the Division of Nutrition Research Coordination (DNRC/NIH). The roles of each of these committees are defined in the NIH Guide announcement dated July 14, 1989 and updated in the NIH Guide announcement dated August 25, 1989. The FOTMDC was formed in December 1988 to deal solely with the approval of requests for test materials; this decision was made to prevent any conflict-of-interest issues related to applicants for test materials.

The application process is initiated by the researcher, who responds to a notice of availability (typically published in the NIH Guide and in scientific journals) by requesting an application form from the DNRC/NIH office. A description of the products is provided with the notice of availability (example, Attachment 2). Completed forms are reviewed by the FOTMDC and recommendations are made on a regular basis (usually quarterly or sooner). The researcher is informed of the Committee decision via a letter from the Program Manager of the BTMP; a copy of the letter is forwarded to the FOTMAC (DNRC/NIH office).

In the case of research that will involve human subjects, the notice of conditional approval will contain the forms required for application to FDA for an IND approval. Once the investigator has been assigned an IND # by FDA, a 30 day waiting period is initiated. The researcher is informed by FDA that a lack of action by the FDA during the 30 day period constitutes approval to use the IND #. A copy of the IND letter from FDA will be forwarded to the FOTMAC (DNRC/NIH office) by the researcher, thereby informing both FOTMAC and the BTMP of the expected approval date.

The distribution process is initiated by a BTM Program representative, who consults with the approved researcher by telephone. A cover letter, QA/QC data sheets, instructions for storage, and a method for formulation of semi-purified animal diets with fish oils are included with each shipment. A copy of the QA/QC Manual is included in the first shipment of test materials.

The overall notification/response process is repeated on a quarterly basis, the details of which will depend upon the scope of test materials offered to the FOTMAC by the BTM Program (Charleston) prior to solicitation.

The first formal NIH notice of availability of the NOAA fish oil test materials appeared in the NIH Guide for Grants and Contracts, May 29, 1987. Requests from researchers and applications for test materials were received in June and the above described decision process was conducted in July. Interviews with the researchers and arrangements for shipment were made during August.

Shipments of soft-gel encapsulated oils (refined, encapsulated steam-deodorized menhaden oil, and bulk fish oil, same material) were made in early September to the NIH-approved researchers. Since that time the FOTMAC has met on numerous occasions, December 1987, March 1988, July 1988, September 1988, December 1988, May 1989 and July 1989 to review the BTM Program and to provide advice and guidance. The test materials have been announced on three other occasions in the NIH Guide for Grants and Contracts (Attachment 2: February 12, 1988: Vol. 17, No. 5; July 14, 1989: Vol. 18, No. 24; August 25, 1989: Vol. 18, No. 29), and in the June 1988 issue of n-3 News (Vol. III, No. 2).

The Test Material Program currently has 83 researchers that have already been approved or are in the final stages for approval of test materials. Of those 83 researchers, 21 have planned human studies and 11 have received approval of their IND application. A status report of the FOTMAC awards is provided in Attachment 3.

Applications may be submitted any time during the year. Copies of the new guidelines may be obtained by contacting:

Richard L. Mowery, Ph.D., Chief
Collaborative Clinical Research Branch
National Eye Institute
Building 31, Room 6A48
Bethesda, Maryland 20892
Telephone: (301) 496-5983

AVAILABILITY OF FISH OIL TEST MATERIALS

P.T. 34; K.W. 0780000, 0780017

National Institutes of Health

This notice supercedes the previous announcement published in the NIH Guide for Grants and Contracts on July 14, 1989 (Vol. 18, No. 24).

SUMMARY AND PURPOSE

TEST MATERIALS CURRENTLY AVAILABLE

- o n-3 ethyl ester concentrate, prepared from menhaden oil, bulk packed or soft-gel encapsulated (80 percent n-3 fatty acids including EPA and DHA)
- o Ethyl esters of olive oil (70 percent oleic), bulk packed or soft-gel encapsulated
- o Deodorized menhaden oil, bulk packed or soft-gel encapsulated
- o Commercial preparations of corn, olive, or safflower oil, soft-gel encapsulated only

PROCESSING AND SPECIFICATIONS OF BIOMEDICAL TEST MATERIALS

o n-3 Ethyl Ester Concentrate

The n-3 ethyl ester concentrate is prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction and short-path distillation. The concentrate contains approximately 80 percent n-3 fatty acid ethyl esters (44 percent EPA, 24 percent DHA, 10-12 percent other n-3 fatty acid ethyl esters), 3 percent C18 (other than n-3), 6 percent C16 and the remainder as other esters. It contains 0.2 mg/g TBHQ as antioxidant, 2 mg/g tocopherols and 2.0 mg/g cholesterol. The concentrate is available in 1 g soft-gel capsules (100 capsules/bottle) or packaged in bulk in quantities suitable to investigators' needs.

o Placebo Ethyl Esters

The ethyl esters of virgin olive oil are prepared by transesterification. The preparation contains approximately 70 percent oleic acid, 13 percent C16, and 15 percent C18 (<1 percent n-3) fatty acid ethyl esters. It contains 0.2 mg/g TBHQ as antioxidant and 2 mg/g tocopherols. The preparation is available in 1 g soft-gel capsules (100 capsules/bottle) or packaged in bulk in quantities suitable to investigators' needs.

o Deodorized Menhaden Oil

Deodorized menhaden oil is prepared from oil that has been winterized and alkali refined; it is processed through a two-stage wiped-film evaporator to remove cholesterol, volatile oxidation products and any traces of organic contaminants. The oil contains approximately 30 percent n-3 fatty acids in the triglyceride form; 14 percent EPA, 8 percent DHA, 8 percent other n-3. It contains 0.2 mg/g TBHQ as antioxidant, 2 mg/g tocopherols and 2.0 mg/g cholesterol. The deodorized oil is available in 1 g soft-gel capsules (100 capsules/bottle) or is packaged in bulk quantities suitable to investigators needs. Special requests for antioxidant-free oil will be considered.

o Placebo Oils

Commercial preparations of corn, olive, and safflower oil have been soft-gel encapsulated to serve as placebos for studies involving encapsulated menhaden oil. These oils contain 0.2 mg/g TBHQ as antioxidant and 2 mg/g tocopherols. The major fatty acids for each oil are: corn (58 percent 18:2n-6, 26 percent 18:1n-9), olive (17 percent 18:2n-6, 57 percent 18:1n-9), safflower (80

percent 18:2n-6, 9 percent 18:1n-9). They are available in 1 g soft-gel capsules (100 capsules/bottle). Although vegetable oils will not be supplied in bulk form, investigators may request analysis of antioxidant and tocopherol levels in vegetable oils that they purchase.

FISH OIL TEST MATERIALS PROGRAM

The Fish Oil Test Materials Program is administered by the Division of Nutrition Research Coordination in the Office of Disease Prevention, NIH. It was established in 1986 through the cooperation of the National Institutes of Health (NIH), the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA), and the National Oceanic and Atmospheric Administration/Department of Commerce (NOAA/DOC). This program has been designed to provide a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers in order to facilitate the evaluation of the role that omega-3 fatty acids play in health and disease.

Fish Oil Test Materials Advisory Committee:

A Fish Oil Test Materials Advisory Committee (FOTMAC) is cochaired by scientific staff from ADAMHA and NIH and is composed of scientists representing the funding agencies (NIH, ADAMHA), the research community, Department of Commerce (DOC), and the Food and Drug Administration (FDA). The FOTMAC provides scientific advice to the DOC regarding the types of materials needed by research scientists, shipping procedures for the materials, and additional quality control and produotion issues. The committee is advisory to the Fish Oil Test Materials Program on general programmatic issues such as future directions and has produced a manual on Good Laboratory Practices for the handling of polyunsaturate materials. In addition, the committee provided guidance to DOC during the production of the Drug Master File submitted to the FDA by the FOTMAC. A manual on Analytical Methods for the Quality Assurance of Fish Oil was produced by the DOC.

Fish Oil Test Materials Distribution Committee:

A Fish Oil Test Materials Distribution Committee (FOTMDC) is composed of NIH and other Federal scientists that do not use these products. The Distribution committee processes the applications received from investigators and advises the DOC of applications that have fulfilled the application process and makes recommendations regarding the distribution of requested materials.

The awarded materials are provided to investigators free of charge. Availability of materials are contingent on DOC/NOAA production capabilities. When prioritization is necessary, the order will be: 1) NIH/ADAMHA funded, 2) other government funded, 3) peer-reviewed, privately funded, 4) NIH/ADAMHA approved, not funded, and 5) other.

To qualify to receive materials described in this announcement the applicant must: 1) have peer-reviewed research indicating the need for the requested materials, and 2) submit a correctly completed application form and a signed waiver of liability. The committee will not be responsible for assessing the scientific merit of the application. Regulations on human subjects and animal research apply. In accordance with federal regulations, an IND number will be required for the use of these materials in human studies. The FOTMAC has established a drug master file at the FDA which includes manufacturing, chemical composition and toxicological data relevant to these products. Investigators using DOC/NOAA materials may reference this file in order to expedite their IND requests.

Requests for materials of amounts greater than 500 kg of vacuum-deodorized menhaden oil and/or 50 kg of n-3 ethyl ester concentrate should not be submitted without prior discussion with the National Marine Fisheries Service - Charleston Laboratory. For further information contact Ms. Patricia Fair at (803) 762-1200.

Test Materials Available in the Future:

Test materials and the relevant application process will be announced in the NIH Guide for Grants and Contracts as new materials become available.

Other Information:

Additional information will be provided the investigator in the form of complete quality assurance data for each lot of test material shipped, general diet preparation information, and instructions for formulation of placebos containing antioxidants balanced to the level in the test material.

INQUIRIES AND APPLICATIONS

Investigators may obtain further information and apply for available fish oil test materials for relevant studies by requesting an application form from:

Ms. Melissa Workman
Program Assistant
Fish Oil Test Materials Program
Division of Nutrition Research Coordination
Building 31, Room 4B63
National Institutes of Health
Bethesda, Maryland 20892
Telephone: (301) 496-2323

MANDATORY USE OF LATEST FELLOWSHIP APPLICATION KITS

P.T. 22. K.W. 0720005 1014006

Division of Research Grants

Beginning with the September 10, 1989, receipt deadline, all applicants for the individual postdoctoral fellowship (F32) or senior fellowship (F33) must use the latest application kits (PHS 416-1, Revised 7/88 or 4/89). Only the 7/88 and 4/89 revisions are acceptable. Submissions on earlier versions of the PHS 416-1 are incompatible with new procedures for the expedited review of fellowship applications and will be returned without review.

AVAILABILITY OF FISH OIL TEST MATERIALS

P.T. 341; K.W. 0780005

National Institutes of Health

SUMMARY AND PURPOSE

FISH OIL TEST MATERIALS PROGRAM

The Fish Oil Test Materials Program was established in 1986 through the cooperation of the National Institutes of Health (NIH), the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA) and the National Oceanic and Atmospheric Administration/Department of Commerce (NOAA/DOC). This program, which is administered by the Division of Nutrition Research Coordination in the Office of Disease Prevention, NIH, was designed to provide a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers in order to facilitate the evaluation of the role that Omega-3 fatty acids play in health and diseases.

FISH Oil Test Materials Advisory Committee:

The Fish Oil Test Materials Advisory Committee (FOTMAC) is cochaired by scientific staff from ADAMHA and NIH and is composed of scientists representing the funding agencies (NIH. ADAMHA), the research community, the Department of Commerce (DOC) and the Food and Drug Administration (FDA). The FOTMAC provides scientific advice to the DOC regarding the types of materials needed by research scientists, shipping procedures for the materials and additional quality control and production issues. The committee is advisory to the Fish Oil Test Materials Program on general programmatic issues, such as future directions, and has produced a Good Lab Practices for Polyunsaturate Handling Manual. In addition, the committee provided guidance to DOC during the production of the Drug Master File that was submitted to the FDA by the FOTMAC. A manual on Analytical Methods for the Quality Assurance of Fish Oil was produced by the DOC.

Fish Oil Test Materials Distribution Committee:

A Fish Oil Test Materials Distribution Committee (FOTMDC) is composed of NIH and other Federal scientists that do not use these products. The Distribution committee processes the applications received from investigators, advises the DOC of applications that have fulfilled the application requirements, and makes recommendations regarding the distribution of requested materials.

The awarded materials are provided to investigators free of charge. Availability of materials are contingent on DOC/NOAA production capabilities. When prioritization is necessary, the order will be: 1) NIH/ADAMHA funded. 2) other government not funded, 3) peer-reviewed, privately funded, 4) NIH/ADAMHA approved, not funded, and 5) other.

REQUIREMENTS

To qualify to receive Materials described in this announcement, the applicant must: 1) be engaged in peer-reviewed research, and 2) submit a correctly completed application form and a signed waiver of liability. The committee will not be responsible for assessing the scientific merit of the application. Regulations on human subjects and animal research apply. In accordance with federal regulations, an IND number will be required for the use of these materials in human studies. The FOTMAC has established a drug master file at the FDA that includes manufacturing, chemical composition, and toxicological

data relevant to these products. Investigators using DOC/NOAA materials may reference this file in order to expedite their IND requests.

TEST MATERIALS CURRENTLY AVAILABLE

- o n-3 ethyl ester concentrate, prepared from menhaden oil, bulk packed or soft-gel encapsulated (80 percent n-3 fatty acids including EPA and DHA)
- o Ethyl esters of olive oil (70 percent oleic), bulk packed or soft-gel encapsulated
- o Deodorized menhaden oil, bulk packed or soft-gel encapsulated
- o Commercial preparations of corn, olive, or safflower oil, soft-gel encapsulated only

PROCESSING AND SPECIFICATIONS OF BIOMEDICAL TEST MATERIALS

o n-3 Ethyl Ester Concentrate

The n-3 ethyl ester concentrate is prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction and short-path distillation. The concentrate contains approximately 80 percent n-3 fatty acid ethyl esters (44 percent EPA, 24 percent DHA, 10-12 percent other n-3 fatty acid ethyl esters), 3 percent C18 (other than n-3), 6 percent C16 and the remainder as other esters. It contains 0.2 mg/g TBHQ as antioxidant, 2 mg/g tocopherols and 2.0 mg/g cholesterol. The concentrate is available in 1 g soft-gel capsules (100 capsules/bottle) or packaged in bulk in quantities suitable to investigators needs.

o Placebo Ethyl Esters

The ethyl esters of virgin olive oil are prepared by transesterification. The preparation contains approximately 70 percent oleic acid, 13 percent C16, and 15 percent C18 (<1 percent n-3) fatty acid ethyl esters. It contains 0.2 mg/g TBHQ as antioxidant and 2 mg/g tocopherols. The preparation is available in 1 g soft-gel capsules (100 capsules/bottle) or packaged in bulk in quantities suitable to investigators needs.

o Deodorized Menhaden Oil

Deodorized menhaden oil is prepared from oil that has been winterized and alkali refined; it is processed through a two-stage wiped-film evaporator to remove cholesterol, volatile oxidation products, and any traces of organic contaminants. The oil contains approximately 30 percent n-3 fatty acids in the triglyceride form; 14 percent EPA, 8 percent DHA, 8 percent other n-3. It contains 0.2 mg/g TBHQ as antioxidant, 2 mg/g tocopherols, and 2.0 mg/g cholesterol. The deodorized oil is available in 1 g soft-gel capsules (100 capsules/bottle) or in bulk quantities suitable to investigators needs. Special requests for antioxidant-free oil will be considered.

o Placebo Oils

Commercial preparations of corn, olive, and safflower oil have been soft-gel encapsulated to serve as placebos for studies involving encapsulated menhaden oil. These oils contain 0.2 mg/g TBHQ as antioxidant and 2 mg/g tocopherols. The major fatty acids for each oil are: corn (58 percent 18:2n-6, 26 percent 18:1n-9), olive (17 percent 18:2n-6, 57 percent 18:1n-9), safflower (80 percent 18:2n-6, 9 percent 18:1n-9). They are available in 1 g soft-gel capsules (100 capsules/bottle). Although vegetable oils will not be supplied in bulk form, investigators may request analysis of antioxidant and tocopherol levels in vegetable oils that they purchase.

Test Materials Available in the Future:

Test materials and the relevant application process will be announced in the NIH Guide for Grants and Contracts as new materials become available.

Other Information:

The investigator will be provided with complete quality assurance data for each lot of test material shipped, general diet preparation information, and instructions for formulation of placebos containing antioxidants balanced to the level in the test material.

INQUIRIES AND APPLICATIONS

Investigators may obtain further information on available fish oil test materials and request an application form from:

Ms. Melissa Workman
Program Assistant
Fish Oil Test Materials Program
Division of Nutrition Research Coordination
Building 31, Room 4B63
National Institutes of Health
Bethesda, Maryland 20892
Telephone: (301) 496-2323

AVAILABILITY OF FISH OIL TEST MATERIALS

P.T. 34; K.W. 0780010

National Institutes of Health

SUMMARY AND PURPOSE

The Fish Oil Test Materials Program has been established through the cooperation of the National Institutes of Health, the Alcohol, Drug Abuse, and Mental Health Administration and the National Oceanic and Atmospheric Administration-Department of Commerce. This program has been designed to provide a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers in order to facilitate the evaluation of the role that omega-3 fatty acids play in health and disease.

TEST MATERIALS CURRENTLY AVAILABLE

o n-3 ethyl ester concentrate
The n-3 ester concentrate is prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction and short-path distillation; the concentrate contains approximately 80 percent n-3 ethyl esters, 3 percent C18 (other than n-3), 6 percent C16 and the remainder as other esters. It is available with antioxidant additions and packaged in quantities suitable to the investigators' needs.

o Encapsulated purified steam-deodorized menhaden oil
o Encapsulated commercial preparations of corn, olive, and safflower oil
These capsules are 1-gram opaque gel capsules, packaged 100 per bottle in tamper-proof sealed brown glass bottles. Alpha-tocopherol and TBHQ antioxidants have been added to these menhaden oil capsules. The vegetable oil capsules have had no antioxidants added. However, these oils do contain endogenous tocopherols.

o Encapsulated purified steam-deodorized menhaden oil
o Encapsulated commercial preparation of corn oil
These capsules are 1 gram clear soft gel, packaged 100 per bottle in tamper-proof sealed brown glass bottles. The antioxidant content of the menhaden oil capsules and the antioxidant content of the corn oil capsules have been balanced for alpa- and gamma-tocopherol and TBHQ. These balanced levels were obtained by adding Eastman Kodak Vitamin E 5-67, GT-1 and Tenox 20A.

o Bulk vacuum-deodorized menhaden oil
The bulk menhaden oil has been winterized, alkali refined and vacuum deodorized. It is available with or without antioxidant additions and is packaged in quantities suitable to the investigators' needs. Corn oil may be purchased by the researcher; provision to quality assure the addition of antioxidants by the researcher will be made.

All products were prepared under a nitrogen blanket and will be supplied with detailed quality assurance data.

In accordance with federal regulations, an IND number will be required for the use of these materials in human studies. The Fish Oil Test Materials Advisory Committee (FOTMAC) will establish a drug master file at the Food and Drug Administration which will include manufacturing, chemical composition and toxicological data relevant to these products. Investigators awarded these materials from the FOTMAC may then reference this file in order to expedite their IND requests. Applications for omega-3 research materials for both human and animal studies will be accepted and processed.

INQUIRIES AND APPLICATIONS

Active investigators may apply for available materials to be used for relevant studies by requesting an application form from:

Nancy Hensler, Program Assistant
Fish Oil Test Materials Program
Building 31, Room 4B63
Nutrition Coordinating Committee
National Institutes of Health
Bethesda, Maryland 20892
Telephone: (301) 496-2323

ATTACHMENT 3.

FOTMAC AWARD SUMMARY - JUNE 1989

101 C.R. Benedict - University of Texas Medical Branch, Galveston, TX
"Effect of Omega-3 Fatty Acids in Coronary Thrombosis"
Sent: 858 kg VDFO

102 C.S. Giam - University of Pittsburgh, Pittsburgh, PA
"Cell Matrix Biology of Fish Liver Carcinogenesis"
Sent: 1 bottle ea. FO, CO, OO, SO

103a Davis A. Otto - Baptist Medical Center, Birmingham, AL
"Omega Fatty Acids and Hepatic Lipid Metabolism"
Sent: 30 bottles ea. OO, SO
70 bottles ea. FO, CO

103b Sent: 14 kg VDFO, 7.5 kg FOE

103c Sent: 20 kg VDFO

103d Sent: 10 kg ea. VDFO, FOE, OOE

104 Gabriel Fernandes - University of Texas, San Antonio, TX
"Influence of Diet on Regulation, Autoimmunity and Aging"
Sent: 72 kg VDFO
120 bottles ea. FO, CO *
*further bottle shipments deferred until researcher submits application for human study and receives IND approval

105 Fredrick C. Kauffman - University School of Medicine, Baltimore, MD
" Metabolic and Developmental Aspects of Mental Retardation"
Sent: 2 bottles ea. FO, CO, OO, SO

106 L.M. Krista - Auburn University, Auburn, AL
"Bursectomy Atherosclerosis and Serum Immunoglobulins in Hyper- and Hypotensive Lines of Turkeys"
Sent: 6 bottles ea. FO, CO
7 kg VDFO

107 Christian T. Campos - University of Minnesota, Minneapolis, MN
"Lipoprotein Effects of Dietary Marine Oil Supplementation"
Sent: 35 kg VDFO

109 A.R.L. Gear - University of Virginia, Charlottesville, VA
"Early Events in Platelet Function"
IND#:
Approved: 10 bottles ea. FO, CO
No record of IND process completion.

110 Samuel K. Martin - Walter Reed Medical Center, Washington, DC
"The Efficacy of Menhaden Oil in Protecting *Plasmodium falaciparum* Malaria Infection"
Sent: 2 kg ea. FOE, OOE

111 Ernest L. Mazzaferri - Ohio State University, Columbus, OH
"N-3 - The Effect of Fatty Acids on Fasting Serum Insulin, Glucose and Peripheral Tissue Responses to Insulin"
IND#:
Approved: 24 bottles FO
Received IND for FO, applied for IND for FOE

112 Christopher J. Hawkey - Queen's Med. Center, Nottingham, England
Cancelled.

113 C.F. Phleger - San Diego State University, San Diego, CA
Sent: 1 bottle FO

114 Larry T. Taylor - Virginia Polytechnic Institute and State University, Blacksburg, VA
Sent: 1 bottle ea. FO, CO, OO, SO

115 J. Martyn Bailey - George Washington University School of Medicine Washington, DC
Sent: 10 bottles ea. FO, CO
3 kg VDFO

116 Hoshitsugi Hokama - University of Hawaii, Honolulu, HA
Sent: 1 bottle ea. FO, CO, OO, SO

117a Lillie M. Boyd - North Carolina Central University, Durham, NC
"The Effects of Menhaden and Mackerel Oil on Arterial Blood Pressure and Cardiac Muscle Lipid Composition and Structure in Spontaneously Hypertensive Rats"
Sent: 2 kg VDFO

117b Sent: 3.5 kg VDFO

120 John S. Parks - Bowman Gray School of Medicine, Winston-Salem, NC
"Atherogenic Properties of Low Density Lipoproteins"
Sent: 90 kg VDFO

122 Henry G. Wilcox - University of Tennessee, Memphis, TN
"Eicosapentanoic Acid and Hepatic Synthesis of Very Low Density Lipoproteins"
Sent: 2 kg VDFO

123 Paul B. Addis - University of Minnesota, St. Paul, MN
"Significance and Inhibition of Lipid Oxidation Products in Foods"
Sent: 3 bottles FO, 1 bottle ea. CO, OO, SO
0.5 kg VDFO

124 Mark Bieber - Best Foods, Union, NJ
Sent: 3 kg VDFO

125 Peter W. Stacpoole - University of Florida, Gainesville, FL
"Nutrient Control of Metabolism in Diabetes"
IND#: 32,346
Sent: 122 bottles ea. FO, SO
0.5 kg VDFO

126 Manfred Steiner - Memorial Hospital of Rhode Island, Nantucket,RI
"Eicosapentanoic Acid: Mechanism of Action on Platelets"
IND#: 32,183
Sent: 300 bottles FO
125 bottles CO

127 Robert S. Lees - Massachusetts Institute of Technology, Cambridge, MA
"Fish Oil Supplements as Adjunctive Therapy of Hypercholesterolemia"
IND #: 32,773
Sent: 150 bottles ea. FOE, OOE

129 Jack Chamberlain - University of the Pacific, San Francisco, CA
"Effects of Long Term Consumption of Fish Oil on Tissue Lipids and Arterial Ultrastructure"
Sent: 5 bottles ea. FO, CO, OO, SO (unbalanced)
5 bottles ea. FO, CO (balanced)
4 kg VDFO

130 Marla Reicks - US Food and Drug Administration, Washington, DC
"Absorption and Metabolism of Triacylglycerol and Ethyl Ester Forms of these Polyunsaturated Fatty Acids"
Sent: 25 g VDFO, 60 g FOE

131 Richard Nelson - University of Illinois College of Medicine, Chicago, IL
"The Effect of Omega-3 Fatty Acids on Colonic C-Kinase *in* Vivo and *in* Vitro"
Sent: 9 kg VDFO, 100 g FOE

132 Syed Q. Alam - Louisiana State University Medical Center, New Orleans, LA
"Dietary Lipids and Cardiac Adenylate Cyclase System"
Sent: 14 kg VDFO, 4 kg FOE

133 Randall Wood - Texas A&M University, College Station, TX
Comparative Analysis
Sent: 1 bottle ea. FO, CO, OO, SO
20 g VDFO
10 g FOE

134 Susan M. Fischer - University of Texas System Cancer Center, Smithville, TX
"The Role of Omega-3 PUFA in Cancer Prevention"
Sent: 3.8 kg VDFO

135 Min-Fu Tsan - Veterans Administration Medical Center, Albany, NY
"Pathophysiology of Pulmonary Vascular Injury and Edema"
Sent: 2 bottles ea. FO, CO
0.5 kg VDFO

136 D. N. Kim - Albany Medical College, Albany, NY
"Fish Oil, Lipoproteins and Atherogenesis"
Sent: 48 kg VDFO

137 David E. Williams - Oregon State University, Corvallis, OR
"Peroxidative Pathways of Carcinogenesis in Trout"
Sent: 9.1 kg VDFO

138 Allen S. Levine - Veterans Administration Medical Center and University of Minnesota, Minneapolis, MN
"Glucose Modulation of Opioid-Induced Feeding"
Sent: 11 kg VDFO

139 Med. O. Adam - Medizinische Poliklinik, Munich, Germany
Approved: 800 bottles ea. FO, OO
Letter sent 12/88 re: current interest.

141 Bonnie Worthington-Roberts - University of Washington, Seattle, WA
"Comparison of Iron Status Between Regular Users of Red Meat and Regular Users of Fish"
IND#: 32,228
Sent: 135 bottles ea. FO, CO

142 Eric M. Scholar - University of Nebraska Medical Center, Omaha, NE
"The Effect of Dietary Fat on Tumor Metastasis"
Approval deferred for additional information: 4 kg VDFO
Letter sent 4/88 requesting clarification of application.

143 Anthony Kafatos - University of Crete, Iraklion Crete
"Greece Epidemiology of Coronary Heart Disease in Cretan Subjects"
Approved 1 bottle ea. FO, CO, OO
Letter sent 1/89 re: current interest, included updated application.

144 Robert E. Anderson - Baylor College of Medicine, Houston, TX
"Role of Lipid Peroxidations in Retinal Degenerations"
Sent: 2 kg VDFO

145 J. Palmer Saunders - University of Texas Medical Branch, Galveston, TX
"Assessment of Changes in Atherosclerotic Plaque Formation"
Sent: 6 bottles ea. FO, CO

146 Suk Y. Oh - University of Utah, Salt Lake City, UT
"Hypercholesterolemic Egg: Enrichment of Omega-3 Fatty Acids into Egg Yolk"
IND#:
Sent: 1 bottle ea. FO, CO, OO, SO (unbalanced)
1 bottle ea. FO, CO (balanced)

147 James C. Fleet - USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA
"Nutritional Modification of Vascular Function: Cellular and Molecular Mechanisms"
Sent: 60 kg VDFO
4 kg FOE

148 Giovanni Galletti - University of Bologna, Bologna, Italy
"Prevention of Thrombosis in Vascular Implants"
Approved 1 kg VDFO
1 kg FOE
Letter Sent 9/88 stating shipment dependent upon completion of application requirements; application sent to reapply for FOE.

149a Orville Levander - USDA Human Nutrition Center, Beltsville, MD
"Anti-Malarial Action of Fish Oils in Vitamin E-Deficient Mice"
Sent: 10 kg VDFO (w/TBHQ only)
10 kg VDFO (w/o TBHQ)
1 kg FOE

149b Sent: 20 kg VDFO (w/TBHQ only)
4 kg FOE

149c Sent: 2 kg VDFO (w/o TBHQ)
2 kg FOE

150 Vicki Kelley - Brigham and Women's Hospital, Boston, MA
"Do N-3 Fatty Acids Alter Interleukin 1 and Tumor Necrosis Factor Gene Expression"
Sent: 3 Kg FOE
1 bottle ea. FOE, OOE

151 James B. Lefkowith - Washington University Medical School, St. Louis, MO
1) "Role of Essential Fatty Acids in Glomerulonephritis"
2) "Role of Lipids in Organ Immunogenicity"
3) "Modulation of the Inflammatory Response by Dietary Polyunsaturated Fatty Acid Manipulation"
Sent: 1 L VDFO
6 L FOE

152 Joseph A. Ontko - Oklahoma Medical Research Foundation, Oklahoma City, OK
"Mechanisms of Cellular Lipid Droplet Mobilization"
Sent: 6 L VDFO

153 Leonard Cohen - American Health Foundation, Valhalla, NY
"N-Nitrosomethylurea-Induced Mammary Cancer and Omega-3 Fatty Acids"
Sent: 45 L VDFO

154a Margaret Craig-Schmidt - Auburn University, Auburn, AL
"Effect of Dietary fish Oil on Murine Mammary Tumorigenesis"
Sent: 12 L VDFO

154b "Role of Dietary Fish Oils and Seed Oils in Human Health"
Sent: 12 L VDFO

155 Arian Zarkower - Pennsylvania State University,
University Park, PA
"Inhibition of Silicolic or Silicotubercular Lesions"
Sent: 80 L VDFO

156 William S. Harris - University of Kansas Medical Center,
Kansas City, MO
"Fish Oil and Lipoprotein Metabolism"
IND#: 32,480
Sent: 144 bottles ea. FOE, OOE

157 George L. Blackburn - New England Deaconess Hospital, Boston, MA
"Dietary Omega-3 PUFAs and Thrombosis After Angioplasty"
IND#: 32,054
Sent: 40 bottles FOE

158a Roger A. Davis - University of Colorado, Denver, CO
"Regulation of Lipoprotein Uptake and Bile Acid Synthesis"
Sent: 1 L VDFO

158b Sent: 1 kg ea. FOE, OOE

159 Laurence A. Harker - Scripps Clinic and Research Foundation
LaJolla, CA
"Dietary N-3 Fatty Acids and Acute Thrombosis and Vascular Healing"
Sent: 28 L FOE

160 David R. Gray - Veterans Administration Medical Center, Long Beach, CA
"The Effects of Omega-3 Fatty Acids on Blood Pressure"
IND#: 32,016
Sent: 120 bottles ea. FO, CO

161 Laura Cook - Iowa State University, Ames, IO
"Effects of Fish Oil on Lipid Metabolism"
Sent: 2 L VDFO
1 L FOE

162 J. Fredrick Cornhill - Laboratory of Experimental Atherosclerosis,
Columbus, OH
"Effects of Fish Oil on Cholesterol"
Sent: 80 kg VDFO

163 John D'Ambola - UCLA Medical Center, Los Angeles, CA
"Hypoxia, Drug Antioxidants, and Lung Macrophage Integrity"
Sent: 4 kg VDFO (w/o TBHQ)

164 Po-Chao Huang - Taiwan University, Taiwan, R.O.C.
"Nutrition Intervention of Hyperlipidemia with Fish Oil"
Sent: 360 bottles FO

165 Robert W. Seerley - University of Georgia, Athens, GA
"Nutrient Requirements of Levels and Sources of Calories and
Environmental Temperatures on Swine"
Sent: 100 L VDFO

166 Claudio Galli - Institute of Pharmacological Science, Milano, Italy
"Comparison of Monosaturated, Polyunsaturated and N-3 Fatty
Acid Diets"
Sent: 20 bottles FO

167 Vincent A. Ziboh - University of California, Davis, CA
"Nutritional Significance of Polyunsaturated Fatty Acids"
Sent: 2 L VDFO
1 L FOE

168 Edward Weiner (Robert Nicolosi) - University of Lowell, Lowell, MA
"Dietary Fat/Cholesterol and Low Density Lipoproteins -
Effect of Dietary Fat on LDL Heterogeneity"
Sent: 5 gal VDFO

169 Herman A.J. Schut - Medical College of Ohio, Toledo, OH
"Carcinogenesis Studies on Heterocyclic Amines"
Sent: 36 L VDFO

170 Aloys L. Tappel - University of California, Davis, CA
"Free Radical Lipid Peroxidation Damage"
Sent: 2 kg VDFO (w/o TBHQ)

171 Michael Bennett - Southwestern Medical School, Dallas, TX
"Immunoregulation of NK Cells"
Sent: 9 kg VDFO (w/o TBHQ)
18 kg VDFO (w/TBHQ)

172 William Connor - Oregon Health Sciences University, Portland, OR
"Dietary Lipids: Effects of Lipid-Lipoprotein Metabolism-
Essentiality of Dietary Omega-3 Fatty Acids in Primates"
Sent: 2 L FOE
2 L OOE

173 Howard R. Knapp - Vanderbilt University, Nashville, TN
"The Pharmacology of Omega-3 Fatty Acids in Man"
IND #: 32,575
Sent: 13.5 kg FOE
5 bottles ea. FOE, OOE

174 Lawrence E. Boerboom - Medical College of Wisconsin, Milwaukee, WI
"Prevention of Vein Graft Atherosclerosis with Fish Oil"
Sent: 378 bottles ea. FOE, OOE

175 Craig Miller - Stanford University, Stanford, CA
"Cardiovascular Surgical Studies of Omega-3"
Sent: 35 L FOE
21 L OOE

176 Robert Vandogen (T.A. Mori) - University of Western Australia, Perth, Australia
"Effects of Fish Diets on Platelet Function"
Approved: 320 bottles FO
Letter sent 5/89 requesting properly signed waiver of liability and letter from Ministry of Health.

177 Richard Moon - IIT Research Institute, Chicago, IL
"Chemoprevention of Mammary Tumors in Rats"
Sent: 92 kg FOE
86 kg OOE

178 Christopher Parrish - University of Toronto, Toronto, Ontario
"Nutritional Control of Lipoprotein Metabolism"
Sent: 3 kg FOE

179 John E. Bauer - University of Florida, Gainesville, FL
"Serum Lipoproteins of Rabbits Fed Fish Oil Diets"
Sent: 1.5 kg VDFO

180 Louis Lippiello - University of Nebraska Medical Center, Omaha, NE
"Lipids in the Pathogenesis of Osteoarthrosis"
Sent: 6 bottles each of FO, FOE, OOE, OO, CO, SO

181 Dennis McClure - Food & Drug Administration, Washington, DC
"Evaluation of High Levels of Fish Oil - Effects on Atherosclerosis"
Sent: 1500 kg VDFO

182 P. Isaac Rabbini - Food & Drug Administration, Washington, DC
"Safety of Fish Oil"
Sent: 70 kg VDFO

183 Roslyn B. Alfin-Slater - UCLA School of Public Health, Los Angeles, CA
"Evaluation of Retinoic Derivative (RO10-1670) in the Treatment of Psoriasis (Compassionate Drug Use)"
IND#:
Approved: 180 bottles FO
Awaiting IND approval.

184 Alexander Leaf - Harvard Medical School, Boston, MA
"Do Fish Oils Prevent Restenosis Post-Coronary Angioplasty?"
IND#: 32,952
Sent: 1417 bottles FOE
1402 bottles COE
15 bottles each of CO, OOE

185 Angelo Scanu - University of Chicago, Chicago IL
"Lipoproteins - Cell Surface Interaction"
IND#: 33,256
Sent: 1 kg VDFO, 400 bottles FO

186 Dwight W. Robinson - Mass General Hospital, Boston, MA
"Prostaglandins in Rheumatic Diseases"
IND#: 32,862
Sent: 2 bottles ea. FOE, OOE

187 Ricardo Uauy - University of Texas Southwestern Medical Center, Dallas, TX
"Are Omega-3 Fatty Acids Essential for Normal Development"
IND#:
Sent: 1.5 kg FOE

188 S. Chi Myung - Lincoln University, Jefferson city, MO
"Effects of Dietary Sodium, Calcium, and Potassium on Hypertension"
Sent: 6 kg VDFO

189 P. V. Subbiah - Rush-Pres.-St Lukes Medical Center Chicago, IL
"Antiatherogenic Actions of Omega-3 Fatty Acids"
IND#:
Approved: 240 bottles FOE
Awaiting IND approval.

DEFINITIONS: VDFO=vaccum-deodorized fish oil; FO=fish oil caps; CO=corn oil caps; COE=corn oil esters, caps or bulk; FOE=fish oil esters, caps or bulk; OO=olive oil caps; OOE=olive oil esters, caps or bulk; SO=safflower oil caps; bottle=100x1g caps.

SECTION III. PROCESSING/CHEMICAL COMPOSITION DATA FOR STEAM-DEODORIZED FISH OIL

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A. GENERAL INFORMATION

1. STARTING MATERIAL SPECIFICATIONS

a. Production of Partially Refined Fish Oil (PRFO)

Menhaden oil of commerce is produced from whole bodies of menhaden by a wet rendering process, typically within 10-15 hours of harvest. The fish are transferred from the vessel's refrigerated holds by conveyors into a cooker and exposed to indirect steam at 96°C for 8-10 minutes to coagulate the protein and rupture the fat cells. Liquids from the cooked fish are then expelled in a bank of hydraulic screw presses and the oil is recovered in a series of centrifuges. The rendered oil is winterized to reduce the proportions of saturated triglycerides by slowly lowering its temperature to 0°C to allow crystallization and removal of higher melting point triglycerides (stearines). Next, the oil is alkali refined by reaction with 4N sodium hydroxide at 90°C to convert free fatty acids to soaps that are then removed from the oil by washing with water. This process also removes phospholipids, nitrogen- and sulphur-containing compounds, and some pigments. Finally, the oils are bleached to improve color by treatment with activated clay. The latter two processes also substantially reduce any heavy metals that may be present. Following these treatments that yield a partially refined oil, approximately 95 percent of the product is shipped to overseas markets for final processing into animal and human food products. Because this oil is produced from the bodies of a fatty fish, there is no danger of vitamins A and D toxicity as there might be in the case of a fish liver oil.

The menhaden industry complies with the guidelines outlined in the FDA guide "Developing a Quality Assurance Program - Contamination of Animal Feedstuffs". This includes screening all articles used in the plant for potential contaminants. These materials include lubricants, pesticides, refrigerants, heat exchange fluids, cleaning materials, laboratory chemicals, paints, and other coatings. The industry requires written assurance from all suppliers that their materials are free of harmful chemicals including PCBs. In addition, all transformers and capacitors are screened to eliminate other potential sources of contamination.

b. Specifications for starting material, PRFO

Partially refined fish oil (PRFO) is the oil obtained from menhaden, ***Brevoortia sp.*** by the process described above in Section III-A.1.a. The oil is a yellow liquid at room temperature and has a characteristic odor and flavor. The specifications for this oil are given in Table 3-1.

TABLE 3-1. QUALITY SPECIFICATIONS FOR PARTIALLY REFINED FISH OIL.

TRIGLYCERIDES, %	>92
EPA, mg/g	>120
DHA, mg/g	>75
FREE FATTY ACIDS, %	<0.2
TRANS ACIDS, %	<5
CHOLESTEROL, mg/g	<5.0
PEROXIDE VALUE, meq/kg	<10.0
IODINE VALUE, g I_2/100g	>160
ANISIDINE VALUE	<50
MOISTURE, ug/g	<500
PCBs, ug/g	<5.0
TOTAL DDT, ug/g	<5.0
TRACE METALS, ug/g:	
Arsenic	<1.0
Cadmium	<1.0
Lead	<1.0
Mercury	<1.0
Selenium	<1.0
SENSORY ATTRIBUTES:	
ODOR (TIO)*	<6.0
FLAVOR (TIF)*	<6.0
OTHER:	
SPECIFIC GRAVITY	0.93
SOLIDIFICATION RANGE	**
SAPONIFICATION VALUE	191-200
UNSAPONIFIABLE MATTER	<1.3%

* TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

** PRFO is a liquid at 5°C or higher.

2. PROCESSING INFORMATION

a. Production of Steam Deodorized Fish Oil (SDFO)

The SDFO was obtained by competitive procurement from the Zapata Haynie Corporation, the world's largest producer of menhaden oil. The oil was deodorized in a Votator-type commercial steam deodorizer under vacuum. The contract required that the supplier have the deodorized oil packaged, while hot, under N_2 in food-grade 55 gal drums. Before sealing, dl-alpha-tocopherol and Tenox 20A (1g each/kg oil) were added as a source of vitamin E and stabilizer, respectively. Tenox 20A contains 20% tertiary butyl hydroquinone (TBHQ), a highly effective stabilizer for unsaturated vegetable oils and edible animal fats.

b. Specifications for Chemicals and Additives Used in the Process

* Tocopherols and antioxidant TBHQ - The tocopherols and the antioxidant TBHQ were added to the steam deodorized fish oil so that the final product concentration was approximately 0.67 mg/g alpha-tocopherol and 0.02% TBHQ. TBHQ was added as a mixture containing the components listed below. The tocopherols and the TBHQ were added to increase the stability and shelf life of the test materials. The actual values in soft-gel capsules vary depending upon a number of factors such as experimental error associated with weighing and mixing the chemicals, as well as analytical methodology. All products were from Eastman Chemical Co., Rochester, NY. These products have been analyzed at the Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils. The products contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
Vitamin E 5-67	672 mg/g d-alpha-tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha.

c. Specifications for the Product, SDFO

Quality specifications for the steam deodorized fish oil include: (1) free fatty acid content of less than 0.15%; (2) iodine value of at least 160; (3) peroxide value no greater than 10 meq/kg oil. Maximum permissible levels of organic contaminants were not specified since steam deodorization is highly effective in reducing these materials to below detectable limits. The successful bidder was required to submit a sample of the steam deodorized oil to the Charleston Laboratory for quality assurance analyses before final acceptance of the low bid. Since these analyses (see below, Section III-A.3) showed the oil to meet specifications, four 55 gal drums of the oil were shipped to the Laboratory where they were placed in storage at -40°C; an additional four 55 gal drums were shipped to a commercial encapsulator for soft-gelatin encapsulation.

3. CHEMICAL COMPOSITION AND QUALITY ASSURANCE

a. Chemical Composition of Encapsulated SDFO

Each encapsulated steam deodorized fish oil product (Chase Chemical Co. and General Nutrition Corporation (GNC)) was assigned a 'lot' number. Each lot is extensively analyzed by the Quality Assurance/ Quality Control Project at the Charleston Laboratory. These analyses include: lipid classification, sterols, fatty acid oxidation products, organics, metals, moisture, sensory attributes, and microbial contamination (Table 3-2a). In addition, a complete fatty acid profile of each oil is obtained by capillary gas chromatography (Table 3-2b). The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

Peroxide value, iodine value, free fatty acid, and moisture content were determined using automated titrators. The peroxide values are well below the quality specifications of 10 meq/kg identified above. Iodine values are above the minimum requirement of 160 g I_2/100g oil.

Antioxidants were analyzed to assure the concentrations added and to provide researchers with a measure of vitamin E activity. The TBHQ content of both products is below the FDA limit of 0.02% in foods.

PCBs and pesticides were analyzed using a capillary gas chromatography method developed in this laboratory; this method is a modification of the EPA and FDA methods. Values are reported for total PCBs (Aroclor 1254) and total DDT (p,p-DDE + o,p-DDE + p,p-DDD + o,p-DDD + p,p-DDT + o,p-DDT).

The products were analyzed for five potentially toxic metals: As, Se, Hg, Cd, and Pb. Pb was present at 0.2 ug/g. All others were below detection limits. The products were also analyzed for eight macroelements which are either of nutritional interest or may potentially be picked up during processing.

The products were subjected to sensory analysis by a trained sensory evaluation panel for total intensity of odor (TIO), total intensity of flavor (TIF), and eleven different odor/flavor attributes. The values reported are based on an unstructured scale 15-cm in length, on which 0 represents "absent" and 15 represents "very strong" presence of a given attribute. The scores obtained on these products represent a rather mild odor/flavor profile. The oils were characterized for color using the Hellige number, which is a standard descriptive value for color intensity of oils.

The gelatin capsules were demonstrated to be free of microbial contaminants using standard FDA methods for determination of *E. Coli* and *Salmonella*.

TABLE 3-2a. CHEMICAL COMPOSITION OF STEAM DEODORIZED FISH OIL.

	TEST MATERIAL	
ANALYSIS TYPE	SDFO A86333A	SDFO A87196A
% TRIGLYCERIDES		
EPA, mg/g	118	120
DHA, mg/g	68	71
TOTAL n-3, mg/g	249	250
FREE FATTY ACIDS, %	0.15	0.29
CHOLESTEROL, mg/g	4.26	4.10
PEROXIDE VALUE, meq/g	1.40	2.48
IODINE VALUE (g I_2/100g)	177.5	179.3
ANISIDINE VALUE	18.16	18.15
ANTIOXIDANT CONTENT:		
a-TOCOPHEROL, mg/g	0.10	1.46
g-TOCOPHEROL, mg/g	<0.05	1.18
TBHQ, mg/g	0.12	0.13
MOISTURE, ug/g	200	436
PCBs, ug/g	0.319	0.15
TOTAL DDT, ug/g	<0.05	<0.05
TRACE METALS, ug/g:		
Arsenic	<0.2	<0.2
Cadmium	<0.12	<0.12
Mercury	<0.5	<0.5
Lead	0.2	*
Selenium	<0.2	<0.2
MACRO ELEMENTS, ug/g:		
Calcium	*	*
Chromium	*	*
Copper	<0.1	<0.1
Iron	5.4	*
Potassium	*	*
Sodium	*	*
Zinc	<0.1	<0.1
SENSORY ATTRIBUTES,		
0-15, 15 MAX INTENSITY:		
ODOR:		
TIO	3.64	2.42
BUTTERY	0.01	0
BEANY	0.11	0.15
RANCID	0.11	0
PAINTY	0.17	0.06
OXIDIZED	0.25	0.05
GRASSY	0.05	0.24
FISHY	0.96	0.72
BITTER	0.03	0
SWEET	0.84	0.08
FRUITY/MELON	0.21	0.09
BURNT	1.29	0.1

TABLE 3-2a. CONTINUED.

FLAVOR:		
TIF	3.76	2.6
BUTTERY	0.33	0.19
BEANY	0.27	0.13
RANCID	0.16	0
PAINTY	0.11	0.07
OXIDIZED	0.17	0.15
GRASSY	0.19	0.32
FISHY	2.08	1.04
BITTER	0.07	0
SWEET	0.73	0.09
FRUITY/MELON	0.14	0.33
BURNT	0.85	0.12
HELLIGE No.:	6	7
BACTERIA		
coliforms	neg	neg
Salmonella	neg	neg

* - not determined

TABLE 3-2b. FATTY ACID COMPOSITION (mg/g) OF STEAM DEODORIZED FISH OIL.

	TEST MATERIAL	
ANALYSIS TYPE	SDFO A86333A	SDFO A87196A
12:0	1.0	1.0
13:0	0.3	0.4
14:0	71.8	72.3
15:0	4.2	4.3
16:0	160.3	162.6
17:0	5.9	6.0
18:0	25.9	26.5
19:0	0.4	0.0**
20:0	1.8	1.8
22:0	1.1	1.0
24:0	0.0	0.0
Total Saturates	271.4	274.6
14:1n7	0.8	0.3
14:1n5	0.2	0.2
16:1n11	0.0	0.0
16:1n9	1.8	1.7
16:1n7	94.1	94.2
16:1n5	3.7	3.6
17:1	0.0	0.0
18:1n11	0.0	0.0
18:1n9	74.9	76.1
18:1n7	26.1	26.4
18:1n5	1.1	0.0
19:1	0.0	0.0
20:1n11+13	1.2	1.2
20:1n9	13.0	13.1
20:1n7	1.5	1.6
20:1n5	2.1	2.1
22:1n11+13	5.9	5.8
22:1n9	2.0	1.9
22:1n7	0.6	0.7
22:1n5	0.0	0.0
24:1n9	2.9	2.6
Total Monoenes	231.9	231.4
16:2n7?	2.3	2.2
16:2n6?	0.6	0.6
16:2n4	12.0	12.0
18:2n9	1.4	2.3
18:2n7	0.0	0.0
18:2n6	10.8	10.7
18:2n4	5.5	5.4
20:2n9	0.0	0.0
20:2n6	1.4	1.3
Total Dienes	34.0	34.5

TABLE 3-2b. CONTINUED.

ANALYSIS TYPE	TEST MATERIAL SDFO A86333A	SDFO A87196A
16:3n4	16.6	16.6
16:3n3	0.7	0.7
16:4n3	0.0	0.0
16:4n1	10.8	10.9
18:3n6	0.0	0.0
18:3n4	3.7	3.5
18:3n3	7.7	7.8
18:4n3	26.2	26.2
18:4n1	3.9	3.9
20:3n6	1.8	1.8
20:3n3	1.2	1.2
20:4n6	7.2	7.2
20:4n3	11.3	11.2
20:5n3	120.4	120.6
21:5n3	5.8	5.4
22:4n6	1.3	1.2
22:5n6	2.0	1.9
22:5n3	18.9	18.9
22:6n3	73.9	74.2
Total Polyenes	347.3	347.7
Total n-3	266.2	266.1
Total n-6	24.4	24.1
n-3/n-6	10.9	11.0
TMTD***	0.0	0.0
Pristanate	0.0	0.0
14:0, ISO	0.0	0.0
14:0, ANTEISO	0.0	0.0
15:0, ISO	2.0	2.0
15:0, ANTEISO	0.0	0.0
17:0, ISO	1.9	1.9
17:0, ANTEISO	0.0	0.0
Phytanate?	3.0	3.0
7MH***	1.2	1.2
7M7H***	0.0	0.0
Total fatty acids	890.8	889.0
Total TG	926.4	924.6

* Fatty acids were tentatively identified by comparison of their RRT values with those of primary and secondary standards and by GC/MS of their methyl esters.

** 0.0 = <0.05 mg/g

*** TMTD=Trimethyltridecanoate, 7MH=7-methylhexadecanoate, 7M7H=7-methyl-7-hexadecanoate.

4. TECHNICAL SUPPORT:

Technical support for the researchers by BTMP personnel includes a discussion of the experimental protocol. Input from the BTMP is focused on the development of the best protocol for the utilization of the test materials while maintaining their high quality. Each researcher approved for the requested amount of Biomedical Test Material (BTM) by the Fish Oil Test Material Distribution Committee (FOTMDC) receives a telephone call soon after receipt of the approval letter. The initial conversation covers a diversity of topics depending upon the research need and protocol. The information in the application is verified in terms of research objective, experimental protocol, and the mode of administration. Information on the availability, composition, storage, and stability data of the BTM is provided. The amount of technical assistance provided to each researcher varies depending upon their specific needs. Some researchers have significant experience in the n-3 research field and require less assistance than those just entering the n-3 arena. Technical support is provided in the forms listed below:

a. Suitability of type and quantity of requested BTM for proposed research protocol.

b. Literature information obtained from the Fish Oil Bibliography.

c. Analytical data, including the quality assurance data.

d. Antioxidants contained in the BTM and balancing of placebo test materials including analysis of placebo oils.

e. Use of control/placebo treatments in experimental designs; flavoring/masking of placebo treatments in double blind trials.

f. Customized packaging and scheduling of shipments of BTM.

Additional information is provided the researcher in the form of printed materials and includes:

g. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

h. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules" (Appendix 4).

i. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil" (Appendix 5).

j. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids.

k. Technical information on the proper storage of each of the test materials to prevent their oxidative deterioration and to ensure maintenance of the high quality of the delivered test material.

l. Technical information is provided in support of IND (Investigative New Drug) applications: The use of BTM in human studies requires the acquirement of an IND number from the Food and Drug Administration. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. This Drug Master File (DMF) containing technical information on the chemical composition, processing, and handling of the specific test materials serves to expedite IND requests for researchers approved for use of the BTM in their studies. The NIH/Fish Oil Test Material Advisory Committee submits the names of approved researchers planning human studies to the FDA to authorize access to the appropriate DMF.

m. NOAA Technical Memorandum NMFS-SEFC-234; "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1).

B. ENCAPSULATION OF STEAM DEODORIZED FISH OIL - First Encapsulation.

Researchers involved in human studies usually use fish oil in the soft-gelatin capsule form. On rare occasion, capsules are utilized to introduce test materials to primates. They are also ideal `containers' for storage of secondary analytical standards.

1. SOFTGEL ENCAPSULATION PROCESS INFORMATION

a. Encapsulation of Refined Oil

The SDFO was encapsulated, under N_2, as No. 20 oblongs, containing 1g oil per capsule, by the Chase Chemical Co., Newark, NJ, using conventional rotary die equipment. At the same time, three vegetable oils were encapsulated for use as placebos in nutritional and clinical studies. These placebo oils are described in Section V in this Drug Master File. Titanium dioxide was added to the gelatin formulation to make the capsules opaque to conceal the color of the oil and preserve the integrity of double blind studies. After washing and drying, as per industry standards, the capsules were packaged and shipped to the Charleston Laboratory where they were stored at 5°C.

An employee of the Laboratory inspected the pertinent sections of the encapsulation facility and observed the encapsulation of one of the three placebo oils. It appeared that good manufacturing practices were being utilized.

2. PACKAGING MATERIALS SPECIFICATIONS

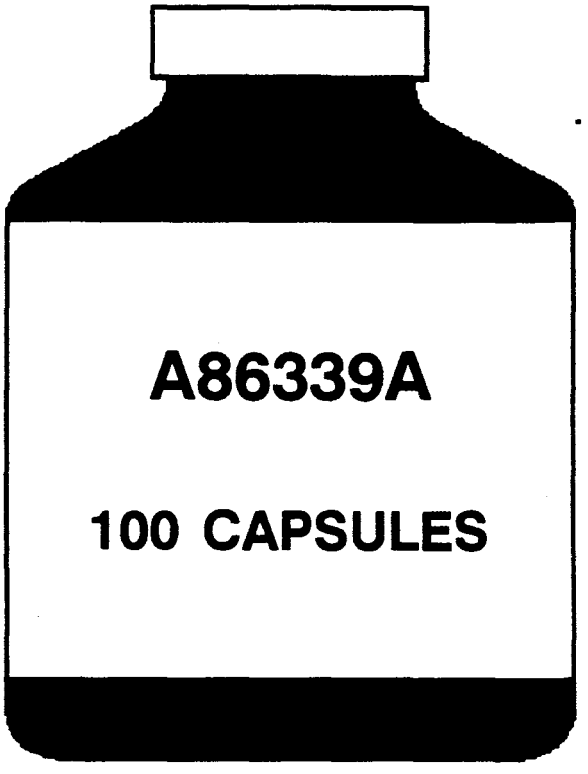
a. Containers

The capsules were packaged in brown glass bottles, 100 - 1 gram capsules/bottle, with screw-cap lids and external tamper-proof seals. The bottles were packaged 24 to a shelf-pack, three shelf-packs to a case. The individual shelf-packs were labeled with the `LOT No.` pertaining to the product contained within, while the outer carton was labeled with the generic term, menhaden fish oil.

b. Labels

An example of the label used on bottles of capsules of steam deodorized fish oil appears in Figure 3-1 and contains the following information: LOT XXXXXXXX, 100 Capsules. Additional labels are sent with these bottles to all users; a cover letter explains that it is the investigators responsibility to use the additional labels in their studies. The additional label contains the following information: BIOMEDICAL TEST MATERIAL, 100 Capsules (1000 mg), Keep refrigerated, "CAUTION: NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE".

FIGURE 3-1. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF STEAM DEODORIZED FISH OIL.



C. ENCAPSULATION OF STEAM DEODORIZED FISH OIL - Succeeding Encapsulation.

1. SOFT GEL ENCAPSULATION PROCESS INFORMATION

a. Introduction

At a later date, one drum of SDFO was withdrawn from freezer storage and encapsulated by General Nutrition Corporation (GNC), Greenville, SC, along with a corn oil placebo (described in Section V), under the direct oversight of a Laboratory staff member. Before encapsulation, additional amounts of tocopherols and TBHQ were added to the fish oil and the placebo, such that both oils contained the same amounts of each component. No coloring agent was used in the gelatin formulation for these capsules. These oils were also encapsulated under N_2. When received at the Charleston Laboratory, the capsules were stored at 5°C.

* Tocopherols and antioxidant TBHQ - The tocopherols (alpha- and gamma-tocopherol) and the antioxidant tertiary butylhydroquinone (TBHQ) were added to the steam deodorized fish oil so that the final product concentration was approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, 0.02% TBHQ. TBHQ was added as a mixture containing the components listed below. The tocopherols and the TBHQ were added to increase the stability and shelf life of the test materials. The actual values in soft-gel capsules vary depending upon a number of factors such as experimental error associated with weighing and mixing the chemicals, as well as analytical methodology. All products are from Eastman Chemical Co., Rochester, NY. These products have been analyzed at Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils. The products contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
Tenox GT-1	Mixed tocopherols (alpha, gamma, delta) 50% to vegetable oil 50%.
Vitamin E 5-67	672 mg/g d-alpha-tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha).

b. Process Description

The rotary die process, invented by R.P. Scherer in 1933, is a continuous process for producing soft gelatin capsules. The rotary die process reduced manufacturing losses to a negligible figure and content variation to ± 1-3%. Capsules are manufactured and partially dried in the following three continuous steps:

* Two gelatin ribbons are prepared, automatically and continuously, and fed, with the fill material, to the encapsulating mechanism.

* The capsules are simultaneously and continuously filled, with the force of the injected fill material causing the gelatin to expand into the die pockets to form the shape of the product, hermetically sealed and automatically cut between two rotary dies.

* The formed capsules are automatically conveyed to and through a solvent wash unit and partially dried in a forced-air tunnel.

The gelatin contains approximately 30 percent water and is heated to a temperature of 37-40°C. The physical characteristics of gelatin are related to the (1) "bloom", a measure of the cohesive strength of the cross-linking, which occurs between gelatin molecules and is proportional to the molecular weight of the gelatin, and (2) viscosity, a measure of the molecular chain length which determines the manufacturing characteristics of the gelatin film.

After the capsules are formed and washed with solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5% of the water. The product is then subjected to drying in a forced air oven (24% R.H., 70°F) for 16 hours which produces a capsule with 6-10% water content at equilibrium.

SDFO was encapsulated with a fill weight of 1,000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. The uniformity of dosage units conforms with USP XXI specifications (pp. 1277-1278). A NMFS BTM program representative inspected and provided oversight at the facilities during encapsulation. It appeared that good manufacturing practices were being utilized at the facility during encapsulation of the test materials. Several modifications of the encapsulation process were performed to minimize contact of the BTM with oxygen and maintain their high quality. The following precautions were taken during encapsulation of the test materials.

* Initial transfer of the fill material from the shipping drum into the process container routinely consists of the drum being lifted and the contents emptied, causing significant mixing of air into the product. This procedure was altered so that the fish oil was transferred, via a transfer line using nitrogen pressure, to the process container flushed with nitrogen. The container was blanketed with nitrogen and closed with an air-tight cover during mixing prior to encapsulation.

* During the packaging of the test materials, nitrogen flushing of the bottled capsules was incorporated at the bottle slow-down point (3 sec flush time), prior to capping.

* Brown glass bottles were used to provide protection for the test materials against exposure to oxygen and light.

These test materials were maintained in a nitrogen atmosphere in temperature controlled containers prior to encapsulation. After encapsulation, the bottled capsules were flushed with nitrogen prior to application of the inner heat seal. Labels were applied on line and are described in Section III-C.2.c of this document. The capsules are stored at 5°C at the Charleston Laboratory.

c. Chemical Composition of Encapsulating Materials

The components used by GNC in formulating the capsule material are gelatin, water, and glycerin. Information regarding the composition of these components is presented below.

* gelatin - Information is provided in Figure 3-2.
* glycerin - Information is provided in Figure 3-3.
* perchloroethylene - meets USP specification requirements for internal use in humans, as stated by the encapsulator.

d. Quality Assurance of Encapsulating Materials

* gelatin - Information is provided in Figure 3-2.
* glycerin - Information is provided in Figure 3-3.
* perchloroethylene wash - The test method consisted of headspace/gas chromatographic analysis for residual perchloroethylene performed on capsules at different stages in the process: directly off the machine; at the end of the dryer prior to the oven; and after 16 and 32 hours of oven drying. Based on headspace analysis, the capsules contain no detectable level of perchloroethylene residue after drying (detection limit = 0.4-0.5 ppm).
* bacterial count on gelatin capsule after curing - After case hardening, the capsules are subjected to microbiological analyses. The encapsulator performs a pathogen screen and standard plate count. All lots have been accepted with a negative pathogenic screening and a standard plate count of <10 CFU/gm. In addition, the NMFS Charleston Laboratory analyzes the gelatin of the encapsulated oils for coliforms and *Salmonella* by the following procedure: (1) pre-enrichment in a non-selective media, (2) selective enrichment in a media, (3) selective plating on brilliant green agar (BGA), MacConkey agar (MAC), and xylose lysine desoxycholate (XLD) agar, (4) identification of suspicious colonies based on colony morphology and (5) confirmed identification using a commercial biochemical test kit (API-20E). The procedures utilized are presented in the QA Methods Manual (Appendix 2).

FIGURE 3-2. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Hide Gelatin Special Blend

RM Listing Designation: Hide Gelatin (P.C.) Blend **RM Code:8626**

Specifications:

Description	: Sheets, flakes, shreds or a coarse to fine powder; faintly yellow or amber in color; the color varying in depth according to particular size; slight characteristic bouillon-like odor in solution
Identification	: Positive by visual inspection and compare to type
Assay/Gel Strength	: 150.0 to 165.0 Bloom
Sieve Analysis	: Not less than 100% through a 16 U.S. Standard Sieve Not less than 1% through a 100 U.S. Standard Sieve
LOD	: Not more than 13%
Arsenic	: Not more than 0.8ppm
Heavy Metals	: Not more than 50 ppm (0.005%) (as Pb)
pH	: 5.8 - 6.2
Residue on Ignition	: Not more than 2.0%
Solubility	: Insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, acetic acid, and hot mixtures of glycerin and water; insoluble in alcohol, chloroform, ether, and fixed and volatile oils.
Viscosity	: 41 $\pm$ 3 mps at 60 degrees C
Dioxide	: Not more than 0.15%
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Pathogens : Negative : Standard Plate Count: Less than 10,000 CFU/gm : Mold & Yeast : Less than 5000 CFU/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot : received. The C of A may be used in place of any of all : testing required above to verify the material meets : specifications.
References	: Vendor Protocol

FIGURE 3-3. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Glycerin - Glycerol 92.09

RM Listing Designation: Glycerin, Natural 99.5% **RM Code:8194**

Specifications:

Description	: Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic.
Identification	: Positive by IR comparison
*Assay	: NLT 95.0% - NMT 101.0%
*Specific Gravity	: NLT 1.249
Solubility	: Miscible with water, alcohol, and methanol
Insoluble	: Chloroform, ether, and fixed & volatile oils
Color	: Clear, colorless liquid / GNC Gardner Scale 1
*Arsenic	: 1.5 ppm
*Heavy Metals	: Limit is 5 ppm
Loss on Ignition	:
Saponification Value:	
*Chloride	: 0.001%
*Sulfate	: NMT 0.002%
*Fatty Acids and Esters: NMT 1 ml of 0.5 sodium hydroxide is consumed	
*Chlorinated compounds: (0.003% of Cl)	
Iodine Value	:
Unsaponifiable Matter (%) :	
Viscosity	: 995 Cps at 25 C, Brookfield apparatus RVT
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Coliforms : Less than 1,000 MPN/gm : Standard Plate Count: Less than 5000 CFU/gm : Mold & Yeast : Less than 500 CFU/gm : Fecal Coliforms : Less than 100 MPN/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot received. The product code number should also be shown on each container and certificate of analysis forwarded to Quality Control.
References	: U.S. Pharmacopeia XXI, p.464

* Designates official USP XXI specification

2. PACKAGING MATERIALS SPECIFICATIONS

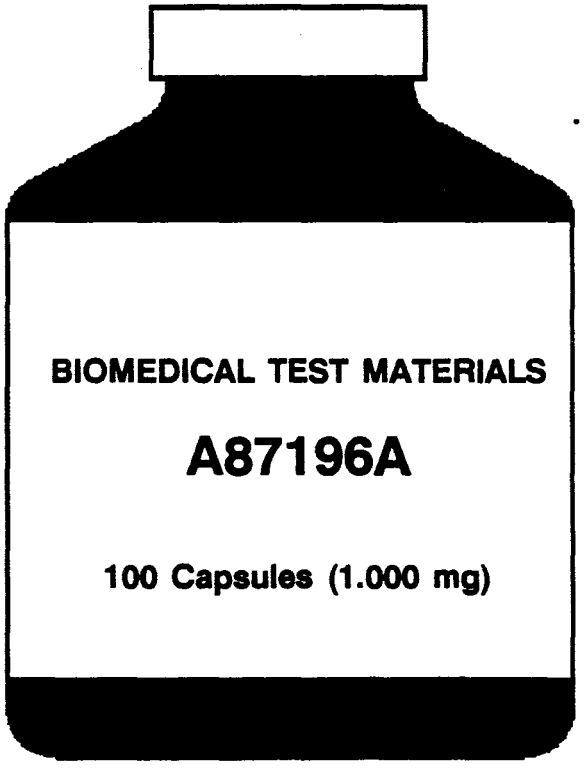
a. Containers

The containers used for bottling capsules of steam deodorized fish oil meet or exceed USP Type III specifications for amber soda-lime glass. These specifications are given on pp. 1223-1224 of Volume XXI of the USP, 1985. The observed light transmission is specified not to exceed 10% at any wavelength in the range of 290nm to 450nm.

b. Labels

An example label used on bottles of capsules of steam deodorized fish oils appears in Figure 3-4 and contains the following information: BIOMEDICAL TEST MATERIAL, LOT XXXXXXXX, 100 Capsules (1,000 mg). Additional labels are sent with these bottles to all users; a cover letter explains that it is the investigators responsibility to use the additional labels in their studies. The additional label contains the following information: BIOMEDICAL TEST MATERIAL, 100 Capsules (1000 mg), Keep refrigerated, "CAUTION: NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE".

FIGURE 3-4. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF STEAM DEODORIZED FISH OIL.



D. STORAGE STABILITY

1. Stability of Encapsulated Oil

A 24-month storage study was conducted in which SDFO in capsules was stored at 5°C. One bottle was randomly selected (every four weeks for the first year and semi-annually thereafter) for a series of assays to determine stability of the product. The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2). The current status of the storage study results (Appendix 4) indicates that there has been little or no change in product quality over 24 months of storage.

SECTION IV. PROCESSING/CHEMICAL COMPOSITION DATA FOR VACUUM-DEODORIZED FISH OIL.

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A. GENERAL INFORMATION

1. STARTING MATERIAL SPECIFICATIONS

a. Production of Partially Refined Fish Oil (PRFO)

Menhaden oil of commerce is produced from whole bodies of menhaden by a wet rendering process, typically within 10-15 hours of harvest. The fish are transferred from the vessel's refrigerated holds by conveyors into a cooker and exposed to indirect steam at 96°C for 8-10 minutes to coagulate the protein and rupture the fat cells. Liquids from the cooked fish are then expelled in a bank of hydraulic screw presses and the oil is recovered in a series of centrifuges. The rendered oil is winterized to reduce the proportions of saturated triglycerides by slowly lowering its temperature to 0°C to allow crystallization and removal of higher melting point triglycerides (stearines). Next, the oil is alkali refined by reaction with 4N sodium hydroxide at 90°C to convert free fatty acids to soaps that are then removed from the oil by washing with water. This process also removes phospholipids, nitrogen- and sulphur-containing compounds, and some pigments. Finally, the oils are bleached to improve color by treatment with activated clay. The latter two processes also substantially reduce any heavy metals that may be present. Following these treatments that yield a partially refined oil, approximately 95 percent of the product is shipped to overseas markets for final processing into animal and human food products. Because this oil is produced from the bodies of a fatty fish, there is no danger of vitamins A and D toxicity as there might be in the case of a fish liver oil.

The menhaden industry complies with the guidelines outlined in the FDA guide "Developing a Quality Assurance Program - Contamination of Animal Feedstuffs". This includes screening all articles used in the plant for potential contaminants. These materials include lubricants, pesticides, refrigerants, heat exchange fluids, cleaning materials, laboratory chemicals, paints, and other coatings. The industry requires written assurance from all suppliers that their materials are free of harmful chemicals including PCBs. In addition, all transformers and capacitors are screened to eliminate other potential sources of contamination.

b. Specifications for starting material, PRFO

Partially refined fish oil (PRFO) is the oil obtained from menhaden, ***Brevoortia sp.*** by the process described in Section A.1.a. The oil is a yellow liquid at room temperature and has a characteristic odor and flavor. The specifications for this oil are given in Table 4-1.

TABLE 4-1. QUALITY SPECIFICATIONS FOR PARTIALLY REFINED FISH OIL.

TRIGLYCERIDES, %	>92
EPA, mg/g	>120
DHA, mg/g	>75
FREE FATTY ACIDS, %	<0.2
TRANS ACIDS, %	<5
CHOLESTEROL, mg/g	<5.0
PEROXIDE VALUE, meq/kg	<10.0
IODINE VALUE, g I_2/100g	>160
ANISIDINE VALUE	<50
MOISTURE, ug/g	<500
PCBs, ug/g	<5.0
TOTAL DDT, ug/g	<5.0
TRACE METALS, ug/g:	
Arsenic	<1.0
Cadmium	<1.0
Lead	<1.0
Mercury	<1.0
Selenium	<1.0
SENSORY ATTRIBUTES:	
ODOR (TIO)	<6.0
FLAVOR (TIF)	<6.0
OTHER:	
SPECIFIC GRAVITY	0.93
SOLIDIFICATION RANGE	**
SAPONIFICATION VALUE	191-200
UNSAPONIFIABLE MATTER	<1.3%

* TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

** PRFO is a liquid at 5°C or higher.

2. PROCESSING INFORMATION

a. Production of Vacuum Deodorized Fish Oil

Partially refined menhaden oil, defined above, was purchased from the Zapata Haynie Corporation, the world's largest producer of menhaden oil. The shipments represent a "draw-down" from a several-thousand-gallon storage tank, constantly being replenished by the producer. When received at the Charleston Laboratory, the oil is placed in storage at 5°C.

Technical operation of the vacuum deodorizer is described in step by step detail in the NOAA Technical Memorandum NMFS-SEFC-234, "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1). The events that occur are as follows. Nitrogen pressure is used to begin the flow of oil from a 55 gal drum of PRFO to the first stage feed pump and thence to the first stage still body. In this stage, rotating teflon blades with diagonal slots spread the oil downward in a thin film along the inner wall of the still. Operating parameters of the first stage are: wiper drive speed, 100 rpm; temperature, 100°C; vacuum, 1 torr. A number of events take place in the first stage. Dissolved gases are removed and the oil is pre-heated, both of which allow the second stage to operate more efficiently. In addition, the temperature and pressure are sufficient to decompose any hydroperoxides present and to distill off short-chained volatile compounds. These volatile compounds condense in a -84°C electric vapor trap and do not enter the vacuum pump.

Before entering the second stage feed pump, the heated oil passes through a sight-glass which permits the operator to balance the pumping rates of the two feed pumps by maintaining a constant level in the sight-glass. Float switches sound an alarm if the sight-glass empties or fills, allowing the operator to make the necessary adjustment in pumping rates. This prevents either stage from flooding or running dry without warning and, thus, protects ultimate oil quality. It also relieves the operator of continual close surveillance of the system. In the second stage still body, rotating carbon blades with diagonal slots propel the oil downward in a thin film on the inner wall of the still body. Operating parameters of the second stage are: wiper drive speed, 100 rpm; temperature, 260°C; vacuum, 0.5 torr. Unlike the first stage, the second stage contains an internal condenser, heated to 150°C by circulating Multitherm, an FDA-approved heat exchange fluid. In the second stage, cholesterol, pesticides, and PCBs are volatilized. Cholesterol (and perhaps some of the organic contaminants as well) condenses on the internal condenser and flows into a waste receiver. Those vapors that do not condense on the internal condenser collect in a glass external condenser or in a -60°C vapor trap that protects the second stage vacuum pump.

The non-volatile triglycerides exit the second stage, pass through a stainless steel heat exchanger where they are cooled to ambient temperature, and enter a 2 gal product collector. When filled, an automatic switch in the collector activates the product pump and the oil is pumped through a 5-10u Teflon filter into an evacuated stainless steel 16 gal pressure vessel, previously charged with the required amounts of antioxidants, for refrigerated or freezer storage. Before the oil is stored, the pressure vessel is placed on a drum roller to thoroughly mix the oil and the antioxidants.

When the oil is needed for shipment to an investigator, it is warmed to ambient temperature and transferred to suitable N_2-flushed containers by pressurizing the storage vessel with N_2. Thus, from the time the oil is pumped out of the 55 gal drum until it enters the shipping container, it is not exposed to air.

b. Specifications for Chemicals and Additives Used in the Process

* Tocopherols and antioxidant TBHQ - The tocopherols (alpha- and gamma-tocopherol) and the antioxidant tertiary butylhydroquinone (TBHQ) were added to the steam deodorized fish oil so that the final product concentration was approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, 0.02% TBHQ. TBHQ is added as a mixture containing the components listed below. The tocopherols and the TBHQ are added to increase the stability and shelf life of the test materials. The actual values in soft-gel capsules will vary depending upon a number of factors such as experimental error associated with weighing and mixing the chemicals, as well as analytical methodology. All products are from Eastman Chemical Co., Rochester, NY. These products have been analyzed at the Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils. The products contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
Tenox GT-1	Mixed tocopherols (alpha, gamma, delta) 50% Vegetable oil 50%
Vitamin E 5-67	672 mg/g d-alpha tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha).

* Gaseous nitrogen - FDA approved and < 5 ppm O_2.

3. EQUIPMENT MAINTENANCE

Special attention has been paid to maintaining the vacuum deodorizer so that test materials of the highest quality possible can be provided. When the still is shut down at the end of each day's operation, it is first filled with fresh oil. This circulates through the product heat exchanger before passing back through both stages until the temperature of the second stage is 45°C or less. The vacuum pumps are then shut off and the system remains under a vacuum overnight. After every four days of operation, the system is heated and flushed with alcoholic alkali, followed by copious volumes of deionized water and absolute ethanol. Quarterly, the system is disassembled and hand cleaned as necessary.

The stainless steel vessels used for collecting and storing the stripped oil also require meticulous cleaning. As soon as a vessel is emptied, the top is removed and a detergent approved by the FDA for use in the food industry is added. The vessel is then filled with very hot water and allowed to stand overnight. The next day, it is scrubbed thoroughly

with fresh detergent, rinsed with hot tap water using a can washer, and finally, rinsed with absolute ethanol. It is left upside down to drain and air dry.

Specific cleaning methods, including frequency, are described in the Production Methods and Safety Manual (Appendix 1). All major production equipment is cleaned with materials described below (undenatured alcohol, deionized water, and alcoholic alkali). All glassware and stainless-steel product receivers are cleaned with detergent (described below) followed by thorough rinsing with tapwater, deionized water, and undenatured absolute ethanol.

* The specification for the detergent used in cleaning the production equipment, including the product receivers, is biodegradable, USDA/FDA approved for use in the food industry. Brand names are "Simple Green" and "Planisol-M".

* Absolute undenatured ethanol - USP grade, anhydrous, 200 proof.

* Deionized water - >16.7 megaohm-cm resistance.

* Alkali (KOH) - technical grade.

4. CHEMICAL COMPOSITION AND QUALITY ASSURANCE OF VDFO

a. Vacuum deodorized fish oil (VDFO)

Vacuum deodorized fish oil (VDFO) is produced from partially refined menhaden oil which is obtained from the small oily menhaden, ***Brevoortia sp.*** (VDFO) is pale yellow in color and has a characteristic odor and flavor. Details of the process by which menhaden oil is refined are provided in Section IV of this Drug Master File. The quality specifications for vacuum deodorized fish oil produced at the Charleston Laboratory are given in Table 4-2.

TABLE 4-2. QUALITY SPECIFICATIONS FOR VACUUM DEODORIZED FISH OIL.

TRIGLYCERIDES, %	>92
EPA, mg/g	>120
DHA, mg/g	>75
FREE FATTY ACIDS, %	<0.2
TRANS ACIDS, %	<5
CHOLESTEROL, mg/g	<5.0
PEROXIDE VALUE, meq/kg	<5.0
IODINE VALUE, g I_2/100g	>160
ANISIDINE VALUE	<50
a-TOCOPHEROL, mg/g	0.5-5.0
g-TOCOPHEROL, mg/g	0.5-5.0
TBHQ, mg/g	0.1-0.2
MOISTURE, ug/g	<500
PCBs, ug/g	<0.5
TOTAL DDT, ug/g	<0.5
TRACE METALS, ug/g:	
Arsenic	<1.0
Cadmium	<1.0
Lead	<1.0
Mercury	<1.0
Selenium	<1.0
SENSORY ATTRIBUTES:	
ODOR (TIO)	<6.0
FLAVOR (TIF)	<6.0
OTHER:	
SPECIFIC GRAVITY	0.93
SOLIDIFICATION RANGE	**
SAPONIFICATION VALUE	191-200
UNSAPONIFIABLE MATTER	<1.3%

* TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

** PRFO is a liquid at 5°C or higher.

b. Chemical Composition and Quality of VDFO

Fish oil obtained from one day's vacuum deodorization run is designated a "batch" for tracking purposes. Batches are pooled until oil is requested for shipment to an investigator, at which time the oil to be shipped is designated a "lot". The vacuum deodorized fish oil is analyzed by lot for purposes of quality assurance. A battery of analyses are performed which fit into several categories: lipid classification, sterols, fatty acid oxidation products, organics, moisture, and sensory attributes. The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2). Table 4-3a presents chemical composition and quality assurance data on a typical lot of vacuum deodorized oil. In addition, a complete fatty acid profile is obtained on each lot using capillary gas chromatography (Table 4-3b).

Peroxide value, iodine value, free fatty acids, and moisture are determined on automated titrators.

Antioxidants are analyzed to assure the concentrations added and to provide researchers with a measure of vitamin E activity. The TBHQ content is measured to assure that the product meets the FDA limit of 0.02% in foods.

PCBs and pesticides are analyzed using a capillary gas chromatography method developed in this laboratory; this method is a modification of the EPA and the FDA methods. Values are reported for total PCBs (Aroclor 1254) and total DDT (p,p-DDE + o,p-DDE + p,p-DDD + o,p-DDD + p,p-DDT + o,p-DDT).

The vacuum deodorized oil is subjected to sensory analysis by a trained sensory evaluation panel for total intensity of flavor (TIF), total intensity of odor (TIO), and eleven different odor/flavor attributes. The values reported are based on an unstructured scale 15-cm in length, on which 0 represents "absent" and 15 represents "very strong" presence of a given attribute. The scores obtained on this product represents a rather mild odor/flavor profile. The oil is characterized for color using the Hellige number, which is a standard descriptive value for color intensity of fish oils.

TABLE 4-3a. CHEMICAL COMPOSITION OF VACUUM DEODORIZED FISH OIL.

	TEST MATERIAL	
ANALYSIS TYPE	VDFO L88232BB	VDFO L89179BB
TRIGLYCERIDE, %		
EPA, mg/g	132	124
DHA, mg/g	83	109
TOTAL n-3, mg/g	292	317
FREE FATTY ACIDS, %	0.04	0.05
TRANS FATTY ACIDS, %	*	<5
CHOLESTEROL, mg/g	2.3	2.2
PEROXIDE VALUE, meq/kg	0.51	0.38
IODINE VALUE, g I_2/100g	176.2	182.7
ANISIDINE VALUE	34.7	42.2
ANTIOXIDANT CONTENT:		
a-TOCOPHEROL, mg/g	1.0	1.1
g-TOCOPHEROL, mg/g	1.2	1.0
TBHQ, mg/g	0.20	0.2
MOISTURE, ug/g	132	201
PCBs, ug/g	<0.05	*
TOTAL DDT, ug/g	<0.05	*
SENSORY ATTRIBUTES,		
0-15, 15 MAX INTENSITY:		
ODOR:		
TIF	3.1	3.35
BUTTERY	0	0
BEANY	0.68	0
RANCID	0	0
PAINTY	0.48	0.63
OXIDIZED	0	0
GRASSY	0	0.13
FISHY	0.25	1.05
BITTER	0.11	0
SWEET	0	0
FRUITY/MELON	0.19	0
BURNT	0	0.15
FLAVOR:		
TIF	3.41	3.85
BUTTERY	0	0
BEANY	0.71	0
RANCID	0	0
PAINTY	0.44	1
OXIDIZED	0	0
GRASSY	0.03	0.48
FISHY	1.13	2.58
BITTER	0.24	0
SWEET	0	0
FRUITY/MELON	0.04	0
BURNT	0	0.28
HELLIGE No.	7	7

* - Not determined

TABLE 4-3b. FATTY ACID COMPOSITION (mg/g) OF VACUUM DEODORIZED FISH OIL.

	TEST MATERIAL	
ANALYSIS TYPE	VDFO L88232BB	VDFO L89179BB
12:0	1.2	1.1
13:0	0.3	0.3
14:0	79.2	63.1
15:0	4.3	4.4
16:0	159.6	151.5
17:0	6.3	6.3
18:0	27.3	25.5
19:0	0.0	0.3
20:0	0.0	2.1
22:0	1.1	1.1
24:0	0.6	0.0
Total Saturates	279.9	247.9
14:1n7	0.3	1.0
14:1n5	0.6	0.3
16:1n11	3.1	0.0
16:1n9	1.8	1.7
16:1n7	94.7	76.2
16:1n5	3.7	3.4
17:1	0.0	0.0
18:1n11	0.0	0.0
18:1n9	69.1	82.7
18:1n7	25.9	25.7
18:1n5	1.8	0.0
19:1	0.0	0.0
20:1n11+13	1.2	1.6
20:1n9	10.7	16.0
20:1n7	1.9	1.6
20:1n5	2.3	2.1
22:1n11+13	0.0	9.3
22:1n9	1.5	2.1
22:1n7	0.8	0.9
22:1n5	0.0	0.0
24:1n9	2.4	3.3
Total Monoenes	221.8	227.8
16:2n7?	2.1	2.0
16:2n6?	0.6	0.6
16:2n4	13.4	10.1
18:2n9	0.0	2.8
18:2n7	0.0	0.0
18:2n6	10.7	12.1
18:2n4	5.6	4.6
20:2n9	0.0	0.0
20:2n6	1.4	1.7
Total Dienes	33.7	33.8

TABLE 4-3b. CONTINUED.

ANALYSIS TYPE	VDFO L88232BB	VDFO L89179BB
16:3n4	17.7	13.9
16:3n3	0.0	0.4
16:4n3	0.0	0.0
16:4n1	12.1	12.1
18:3n6	0.0	0.0
18:3n4	4.6	2.7
18:3n3	8.4	10.2
18:4n3	26.9	32.2
18:4n1	3.1	3.5
20:3n6	2.3	1.5
20:3n3	1.0	1.4
20:4n6	7.8	5.6
20:4n3	12.5	13.9
20:5n3	131.6	124.5
21:5n3	6.5	5.8
22:4n6	1.5	1.1
22:5n6	2.4	2.5
22:5n3	22.9	19.9
22:6n3	82.7	108.8
Total Polyenes	349.7	393.8
Total n-3	292.5	317.1
Total n-6	26.7	24.5
n-3/n-6	11.0	12.9
TMTD***	0.0	0.0
Pristanate	0.0	0.0
14:0, ISO	0.0	0.0
14:0, ANTEISO	0.0	0.0
15:0, ISO	2.1	2.3
15:0, ANTEISO	0.6	0.0
17:0, ISO	1.9	2.1
17:0, ANTEISO	0.9	0.0
Phytanate?	0.0	2.2
7MH***	1.1	1.0
7M7H***	0.0	0.0
Total fatty acids	901.0	903.1
Total TG	937.0	939.2

* Fatty acids were tentatively identified by comparison of their RRT values with those of primary and secondary standards and by GC/MS of their methyl esters.

** 0.0 = <0.05 mg/g

*** TMTD=Trimethyltridecanoate, 7MH=7-methylhexadecanoate, 7M7H=7-methyl-7-hexadecanoate.

5. TECHNICAL SUPPORT

Technical support for the researchers by BTMP personnel includes a discussion of the experimental protocol. Input from the BTMP is focused on the development of the best protocol for the utilization of the test materials while maintaining their high quality. Each researcher approved for the requested amount of Biomedical Test Material (BTM) by the Fish Oil Test Material Distribution Committee (FOTMDC) receives a telephone call soon after receipt of the approval letter. The initial conversation covers a diversity of topics depending upon the research need and protocol. The information in the application is verified in terms of research objective, experimental protocol, and the mode of administration. Information on the availability, composition, storage, and stability data of the BTM is provided. The amount of technical assistance provided to each researcher varies depending upon their specific needs. Some researchers have significant experience in the n-3 research field and require less assistance than those just entering the n-3 arena. Technical support is provided in the forms listed below:

a. Suitability of type and quantity of requested BTM for proposed research protocol.

b. Literature information obtained from the Fish Oil Bibliography.

c. Analytical data, including the Quality Assurance data.

d. Antioxidants contained in the BTM and balancing of placebo test materials including analysis of placebo oils.

e. Use of control/placebo treatments in experimental designs; flavoring/masking of placebo treatments in double blind trials.

f. Customized packaging and scheduling of shipments of BTM.

Additional information is provided the researcher in the form of printed materials and includes:

g. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

h. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules" (Appendix 4).

i. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil" (Appendix 5).

j. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids.

k. Technical information on the proper storage of each of the test materials to prevent their oxidative deterioration and to ensure maintenance of the high quality of the delivered test material.

1. Technical information is provided in support of IND (Investigative New Drug) applications: The use of BTM in human studies requires the acquisition of an IND number from the Food and Drug Administration. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. This Drug Master File (DMF) containing technical information on the chemical composition, processing, and handling of the specific test materials serves to expedite IND requests for researchers approved for using the BTM in their studies. The NIH/Fish Oil Test Material Program (FOTMP) office submits the names of approved researchers planning human studies to the FDA to authorize access to the appropriate DMF.

m. NOAA Technical Memorandum NMFS-SEFC-234; "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1).

B. ENCAPSULATION OF VACUUM DEODORIZED FISH OIL

Researchers involved in human studies usually use fish oil in the soft-gelatin capsule form. On rare occasion, capsules are utilized to introduce test materials to primates. They are also ideal 'containers' for storage of secondary analytical standards.

1. SOFT GELATIN ENCAPSULATION PROCESS INFORMATION

a. Introduction

The rotary die process, invented by R.P. Scherer in 1933 is a continuous process for producing soft gelatin capsules. The rotary die process reduced manufacturing losses to a negligible figure and content variation to $\pm$ 1-3%. Capsules are manufactured and partially dried in the following three continuous steps:

* Two gelatin ribbons are prepared, automatically and continuously, and fed with the product to the encapsulating mechanism.
* The capsules are simultaneously and continuously filled, with the force of the injected product causing the gelatin to expand into the die pockets to form the shape of the product, hermetically sealed and automatically cut between two rotary dies.
* The formed capsules are automatically conveyed to and through a solvent wash unit and partially dried in a forced-air tunnel.

The gelatin contains approximately 30 percent water and is heated to a temperature of 37-40°C. The physical characteristics of gelatin are related to the (1) "bloom", a measure of the cohesive strength of the cross-linking, which occurs between gelatin molecules, and is proportional to the molecular weight of the gelatin, and (2) viscosity, a measure of the molecular chain length which determines the manufacturing characteristics of the gelatin film.

After the capsules are formed and washed with solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5% of the water. The product is then subjected to drying in a forced air oven (24% R.H., 70°F) for 16 hours which produces a capsule with 6-10% water content at equilibrium.

b. **Process Description**

Vacuum deodorized fish oil is encapsulated at General Nutrition Corporation (GNC), Greenville, SC with a fill weight of 1,000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. The uniformity of dosage units utilized conforms with USP XXI specifications (pp. 1277-1278). A NMFS BTM program representative inspects and provides oversight at encapsulation facilities during encapsulation of the vacuum deodorized fish oil. It appears that good manufacturing practices are being utilized at the facility during encapsulation of the vacuum deodorized fish oil. Several modifications of the encapsulation process are performed to minimize contact of the BTM with oxygen and maintain the high quality of the BTM. The following precautions are taken during encapsulation of the test materials.

* Initial transfer of the vacuum deodorized fish oil from the drum into the container routinely consists of the drum being lifted and the contents emptied, causing significant mixing of air into the product. This procedure is altered so that the contents are transferred, via a transfer line using nitrogen pressure, to a container flushed with nitrogen. The container is blanketed with nitrogen and closed with an air-tight cover during mixing and prior to encapsulation.
* During the packaging of the vacuum deodorized fish oil, nitrogen flushing of the bottled capsules is incorporated at the bottle slow down point (3 sec flush time), prior to capping.
* Brown, polyethylene bottles are used to provide the best protection for the test materials while facilitating shipping of the product.

These capsules are maintained in a nitrogen atmosphere in temperature controlled containers prior to bottling. After bottling, the capsules are flushed with nitrogen prior to application of the inner heat seal. Labels are applied on line and are described in Section IV-B.2.b of this document. The capsules are stored at 5°C at the Charleston Laboratory.

c. **Chemical Composition of Materials Utilized in the Encapsulation Process**

The components used by GNC in formulating the capsule material are gelatin, water, and glycerin. Information regarding the composition of these components is presented below.

* gelatin - Information is provided in Figure 4-1.
* glycerin - Information is provided in Figure 4-2.
* perchloroethylene - meets USP specification requirements for internal use in humans, as stated by the encapsulator, GNC.

d. **Quality Assurance of Encapsulating Materials**

* gelatin - Information is provided in Figure 4-1.
* glycerin - Information is provided in Figure 4-2.

* perchloroethylene wash - The test method consisted of headspace/gas chromatographic analysis for residual perchloroethylene performed on capsules at different stages in the process: directly off the machine; at the end of the dryer prior to the oven; and after 16 and 32 hours of oven drying. Based on headspace analysis, the capsules contain no detectable level of perchloroethylene residue after drying (detection limit = 0.4-0.5 ppm).

* bacterial count on gelatin capsule after curing - After case hardening, the capsules are subjected to microbiological analyses. The encapsulator performs a pathogen screen and standard plate count. All lots have been accepted with a negative pathogenic screening and a standard plate count of <10 CFU/gm. In addition, the NMFS Charleston Laboratory analyzes the gelatin of the encapsulated oils for coliforms, and *Salmonella* using the procedures presented in the QA Methods Manual (Appendix 2).

FIGURE 4-1. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Hide Gelatin Special Blend

RM Listing Designation: Hide Gelatin (P.C.) BlendRM Code:8626

Specifications:

Description	: Sheets, flakes, shreds or a coarse to fine powder; faintly yellow or amber in color; the color varying in depth according to particular size; slight characteristic bouillon-like odor in solution
Identification	: Positive by visual inspection and compare to type
Assay/Gel Strength	: 150.0 to 165.0 Bloom
Sieve Analysis	: Not less than 100% through a 16 U.S. Standard Sieve Not less than 1% through a 100 U.S. Standard Sieve
LOD	: Not more than 13%
Arsenic	: Not more than 0.8ppm
Heavy Metals	: Not more than 50 ppm (0.005%) (as Pb)
pH	: 5.8 - 6.2
Residue on Ignition	: Not more than 2.0%
Solubility	: Insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, acetic acid, and hot mixtures of glycerin and water; insoluble in alcohol, chloroform, ether, and fixed and volatile oils.
Viscosity	: 41 $\pm$ 3 mps at 60 degrees C
Dioxide	: Not more than 0.15%
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Pathogens : Negative : Standard Plate Count: Less than 10,000 CFU/gm : Mold & Yeast : Less than 5000 CFU/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot : received. The C of A may be used in placed of any of all : testing required above to verify the material meets : specifications.
References	: Vendor Protocol

FIGURE 4-2. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Glycerin - Glycerol 92.09

RM Listing Designation: Glycerin, Natural 99.5%RM Code:8194

Specifications:

Description	: Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic.
Identification	: Positive by IR comparison
*Assay	: NLT 95.0% - NMT 101.0%
*Specific Gravity	: NLT 1.249
Solubility	: Miscible with water, alcohol, and methanol
Insoluble	: Chloroform, ether, and fixed & volatile oils
Color	: Clear, colorless liquid / GNC Gardner Scale 1
*Arsenic	: 1.5 ppm
*Heavy Metals	: Limit is 5 ppm
Loss on Ignition	:
Saponification Value:	
*Chloride	: 0.001%
*Sulfate	: NMT 0.002%
*Fatty Acids and Esters: NMT 1 ml of 0.5 sodium hydroxide is consumed	
*Chlorinated compounds: (0.003% of Cl)	
Iodine Value	:
Unsaponifiable Matter (%) :	
Viscosity	: 995 Cps at 25 C, Brookfield apparatus RVT
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Coliforms : Less than 1,000 MPN/gm : Standard Plate Count: Less than 5000 CFU/gm : Mold & Yeast : Less than 500 CFU/gm : Fecal Coliforms : Less than 100 MPN/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot received. The product code number should also be shown on each container and certificate of analysis forwarded to Quality Control.
References	: U.S. Pharmacopeia XXI, p.464

* Designates official USP XXI specification

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

The containers used for bottling capsules of vacuum deodorized fish oil are amber high density linear polyethylene bottles as described in Figure 4-3.

FIGURE 4-3. GENERAL SPECIFICATIONS FOR PLASTIC BOTTLES.

REVISED: JUNE 14, 1985

MEETS USP III CONTAINER SPECIFICATIONS PAGES 1238-1240.

Amber Plastic Bottles

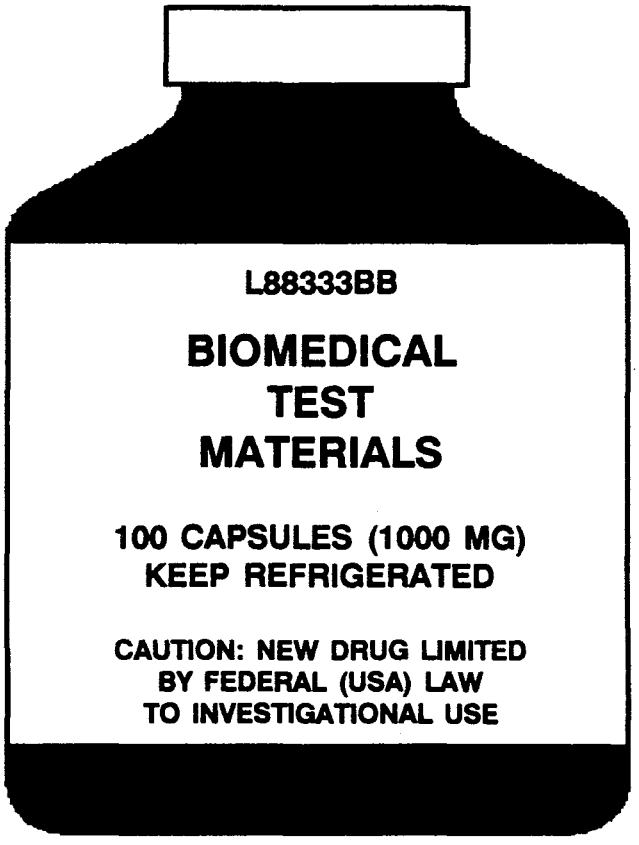
a. Resin - high density, linear polyethylene
b. Pigment - Iron Oxide
c. Mold Release Compound - Zinc Stearate 0.15 percent
d. Flame Treated
e. Style M Necks

	MINIMUM WALL THICKNESS
<500 cc	0.025"
500 cc	0.035"
625 cc	0.035"
750 cc	0.038"
950 cc	0.040"
1300 cc	0.047"

b. Labels

An example of the label used on bottles of capsules of vacuum deodorized fish oil appears in Figure 4-4 and contains the following information: BIOMEDICAL TEST MATERIALS, Lot XXXXXXXX, 100 Capsules (1000 mg), Keep Refrigerated, "CAUTION: NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE".

FIGURE 4-4. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF VACUUM DEODORIZED FISH OIL.



3. STORAGE STABILITY

A storage stability study carried out on steam deodorized fish oil, encapsulated in gelatin (Appendix 4), demonstrated that the product was stable for at least 12 months. Continued analyses of these capsules, performed after the publication of the 12-month study, showed no change in oil quality after 24 months. The storage stability study conducted on vacuum deodorized fish oil in soft gelatin capsules will therefore be a minimal study, analyzing only 0, 12 and 24 month time points. The data for VDFO stored at 5°C is summarized in Table 4-4.

TABLE 4-4. STORAGE STABILITY OF SOFTGEL CAPSULES OF VACUUM DEODORIZED FISH OIL.

	MONTHS STORED		
ANALYSIS TYPE	0	12	24*
EPA, mg/g	136	140	
DHA, mg/g	85	84	
TOTAL n-3, mg/g	308	305	
PEROXIDE VALUE	1.22	0.97	
ANISIDINE VALUE	37.0	-	
a-TOCOPHEROL, mg/g	0.9	0.9	
g-TOCOPHEROL, mg/g	1.0	1.0	
SENSORY ATTRIBUTES, 0-15, 15 MAX INTENSITY:			
ODOR:			
TIO	3.10	4.07	
BUTTERY	0	0	
BEANY	0.68	0	
RANCID	0	0	
PAINTY	0.48	0.77	
OXIDIZED	0	0	
GRASSY	0	0	
FISHY	0.25	2.1	
BITTER	0.11	0	
SWEET	0	0	
FRUITY/MELON	0.19	0	
BURNT	0	0.17	
DECOMPOSITION	0	0.23	
FLAVOR:			
TIF	3.41	4.92	
BUTTERY	0	0	
BEANY	0.71	0.62	
RANCID	0	0	
PAINTY	0.44	0	
OXIDIZED	0	0.08	
GRASSY	0.03	0	
FISHY	1.13	3.48	
BITTER	0.24	0	
SWEET	0	0.27	
FRUITY/MELON	0.04	0	
BURNT	0	0	
DECOMPOSITION	0	1.6	

*Data to be collected 12/90.

C. BULK PACKAGING OF VACUUM DEODORIZED FISH OIL

Researchers conducting animal experimentation primarily utilize vacuum deodorized fish oil in the bulk form. The refined oil is custom packaged consistent with the research protocol.

1. PROCESS INFORMATION

a. Introduction

One of the simplest techniques for the storage and dispensing of liquids is the use of screw-cap bottles. Screw-cap bottles are satisfactory for dispensing single samples and product stability will be maintained if the material is bottled under nitrogen and stored at low temperatures. However, because bottles are not perfectly amenable to uses requiring multiple-dose dispensing, they require nitrogen purging between uses, to maintain an inert atmosphere. The BTM is custom packaged in a manner consistent with its use in the research protocol. The objective of custom packaging is directed at providing the researcher with the quantity of material that will facilitate opening the container a minimum number of times. For instance, the BTM is aliquoted into quantities that facilitate the mode of administration, such as the quantity utilized in the formulation of diet at one time, or the amount of material to be used in gavage studies for a one week period. The Charleston laboratory will, on occasion, conduct a simulated trial of a research protocol to obtain data on the product stability and chemical characteristics when exposed to such a protocol.

b. Process Description

The bulk vacuum deodorized fish oil is held in stainless steel tanks under a vacuum or 55 gal food-grade steel drums under a nitrogen atmosphere. The vacuum deodorized fish oil is stored at 5°C for a 6 month period during which the product is used for research requests. At the end of the 6 month period any remaining vacuum deodorized fish oil is returned to production for reprocessing. The materials are transferred via nitrogen pressure to the specified size and quantity of containers for each researcher. The containers are flushed with nitrogen prior to, and during the filling process. The bulk oil is well-blanketed with nitrogen prior to capping. The bottles are capped tightly, fastened with tape, placed in plastic bags and tied, in case of spillage during shipping.

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

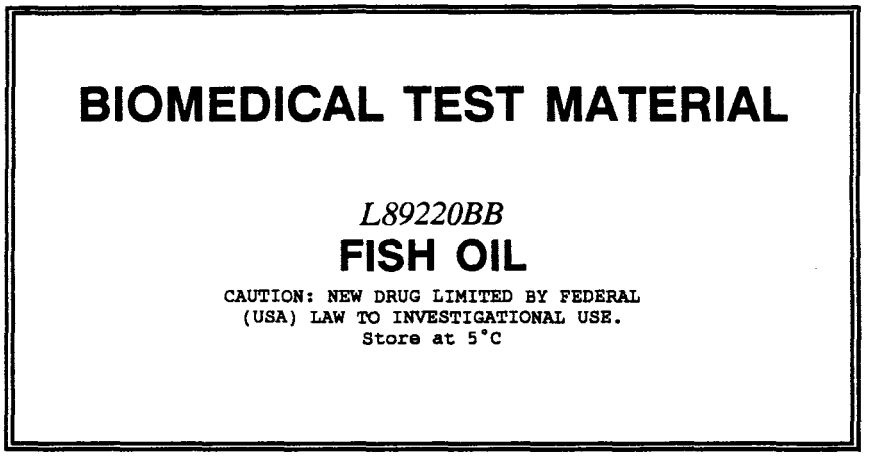
All of the plastic containers presently used for shipment of bulk oil are FDA approved for food use. The high density polyethylene containers, used for shipment of bulk oil, are those supplied by Nalgene Corporation, Rochester, NY. The following stock numbers reflect these Nalgene amber, polyethylene food-grade containers:

2004-8125 (4 ml)
2004-9125 (4 ml)
2004-9025 (8 ml)
2004-9050 (15 ml)
2004-0001 (30 ml)
2004-0002 (60 ml)
2009-0004 (125 ml)
2009-0008 (250 ml)
2009-0016 (500 ml)
2009-0032 (1000 ml)
2009-0064 (2000 ml)

b. Labels

Labels currently are being generated using computer fonts and a laser printer, photocopied onto self-adhesive labels and applied to the bulk containers. The label contains the following information: BIOMEDICAL TEST MATERIAL, Lot XXXXXXXX, Store at 5°C, "CAUTION: NEW DRUG LIMITED BY FEDERAL USE (USA) LAW TO INVESTIGATIONAL USE". An example label used on the bulk containers of vacuum deodorized fish oil is shown in Figure 4-5.

FIGURE 4-5. EXAMPLE LABEL FOR BULK CONTAINERS OF VACUUM DEODORIZED FISH OIL.



3. STORAGE STABILITY

A storage stability study has been initiated in which vacuum deodorized fish oil, containing alpha-tocopherol and TBHQ antioxidant, are being stored at -40°C in 100 ml aliquots in Nalgene thick-walled polyethylene bottles; and at 5°C in both Nalgene thick-walled polyethylene bottles and thin-walled polyethylene bottles. Samples will be analyzed quarterly over a period of two years to determine the stability of the vacuum deodorized fish oil in terms of fatty acid composition, iodine value, free fatty acid content, peroxide value, anisidine value, antioxidant content, and sensory attributes. A summary of the data is presented in Table 4-5.

TABLE 4-5. STORAGE STABILITY OF BULK PACKED VACUUM DEODORIZED FISH OIL.

TIME (months)	TREATMENT	PV	AV	a-Toc	g-Toc	EPA	DHA	TOT n-3
0		0.43	34.7	0.6	0.9	134	85	298
3	thick -40 C	0.44	-	-	-	-	-	-
	thick 5 C	2.75	-	-	-	-	-	-
	thin 5 C	3.06	-	-	-	-	-	-
6	thick -40 C	0.43	33.8	0.8	1.1	134	85	298
	thick 5 C	9.12	33.0	0.8	1.1	133	84	295
	thin 5 C	10.08	33.4	0.8	1.1	134	85	297
9	thick -40 C	0.42	-	-	-	-	-	-
	thick 5 C	11.43	-	-	-	-	-	-
	thin 5 C	15.71	-	-	-	-	-	-
12	thick -40 C	0.26	35.8	0.8	1.1	130	81	289
	thick 5 C	16.37	37.1	0.8	1.1	129	81	288
	thin 5 C	22.62	37.4	0.8	1.1	128	80	284
18*	thick -40 C							
	thick 5 C							
	thin 5 C							
24	thick -40 C							
	thick 5 C							
	thin 5 C							

* Data to be collected 2/90.

SECTION V. PROCESSING/CHEMICAL COMPOSITION DATA FOR COMMERCIALLY PRODUCED PLACEBO OILS.

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3. STORAGE STABILITY

D. **BULK PACKAGING of VEGETABLE OILS**

A. GENERAL INFORMATION

1. STARTING MATERIAL SPECIFICATIONS

a. Corn Oil

Corn oil is the refined fixed oil obtained from the embryo of ***Zea mays*** Linne' (Fam. Gramineae). It is a clear, light yellow, oily liquid. It has a faint, characteristic odor and taste. It is slightly soluble in alcohol and miscible with ether, chloroform, benzene and petroleum ether. The quality specifications for this oil are included in Table 5-1.

b. Olive Oil

Olive oil is the fixed oil obtained from the ripe fruit of ***Olea europaea*** Linne' (Fam. Oleaceae). It is pale yellow, or light greenish yellow, oily liquid having a slight characteristic odor and taste, with a faintly acrid after-taste. It is slightly soluble in alcohol and is miscible with ether, chloroform, and carbon disulfide. The quality specifications for this oil are included in Table 5-1.

c. Safflower Oil

Safflower oil is the refined fixed oil obtained from the seed of ***Carthamus tinctorius*** Linne' (Fam. Compositae). It is a clear, light yellow, oily liquid. It has a faint, characteristic odor and taste. It is slightly soluble in alcohol and miscible with ether, chloroform, benzene and petroleum ether. The quality specifications for this oil are included in Table 5-1.

TABLE 5-1. QUALITY SPECIFICATIONS FOR VEGETABLE OILS.

ANALYSIS TYPE	VEGETABLE OIL		
	Corn	Olive	Safflower
TRIGLYCERIDES, %	>95	>95	>95
16:0, %	8-12	9-17	6-7
18:1n-9, %	19-49	50-84	9-14
18:2n-6, %	34-62	4-18	76-81
FREE FATTY ACIDS, %	<0.2	<0.2	<0.2
CHOLESTEROL, mg/g	0	0	0
PEROXIDE VALUE, meq/kg	<10.0	<10.0	<10.0
IODINE VALUE, g I_2/100g	102-130	79-88	135-150
ANISIDINE VALUE	<20	<20	<20
a-TOCOPHEROL, mg/g	0.1-1.0	0.1-1.0	0.1-1.0
g-TOCOPHEROL, mg/g	0.1-1.0	0.05-0.5	0.05-0.5
MOISTURE, ug/g	<500	<500	<500
PCBs, ug/g	<0.5	<0.5	<0.5
TOTAL DDT, ug/g	<0.5	<0.5	<0.5
TRACE METALS, ug/g:			
Arsenic	<1.0	<1.0	<1.0
Cadmium	<1.0	<1.0	<1.0
Lead	<1.0	<1.0	<1.0
Mercury	<1.0	<1.0	<1.0
Selenium	<1.0	<1.0	<1.0
SENSORY ATTRIBUTES:			
ODOR (TIO)	<4.0	<4.0	<4.0
FLAVOR (TIF)	<4.0	<4.0	<4.0
OTHER:			
SPECIFIC GRAVITY	.914-.921	.910-.915	.919-.924
SOLIDIFICATION RANGE	-10 to -6°C	-8 to -3°C	-18 to -16°C
SAPONIFICATION VALUE	187-193	190-195	186-194
UNSAPONIFIABLE MATTER	<1.5%	<1.5%	<1.5%

* TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

2. PROCESSING INFORMATION

a. Addition of Tocopherols and Antioxidants

Tocopherols and antioxidant TBHQ - The tocopherols (alpha- and gamma-tocopherol) and the antioxidant tertiary butylhydroquinone (TBHQ) are added to the vegetable oils so that the final product has approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, 0.02% TBHQ. TBHQ is added as a mixture containing the components listed below. The tocopherols and TBHQ are added to increase the stability and shelf life of the test materials and to achieve a balance of these components between the placebo oil and the VDFO. The objective is to attain approximately 1.0 mg/g for both alpha- and gamma-tocopherol. The actual values in soft-gel capsules will vary depending upon a number of factors such as experimental error associated with weighing and mixing the chemicals, as well as analytical methodology. Tocopherols contained in the vegetable oils are those naturally occurring and those added to attain a balanced level with the complimentary fish oil test material; the added components consist of Vitamin E 5-67, Tenox GT-1, and Tenox 20A (all products are from Eastman Chemical Co., Rochester, NY). These products have been analyzed at the Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils.

b. Specifications for Chemicals and Additives

* Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
* Tenox GT-1	Mixed tocopherols (alpha, gamma, delta) 50% Vegetable oil 50%.
* Vitamin E 5-67	672 mg/g d-alpha-tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha-tocopherol).
* Gaseous nitrogen	FDA approved and < 5 ppm O_2.

3. EQUIPMENT MAINTENANCE

Specific cleaning methods, including frequency, are described in the Production Methods and Safety Manual (Appendix 1). All major production equipment is cleaned with materials described below (undenatured alcohol, deionized water, and alcoholic alkali). All glassware and stainless-steel product receivers are cleaned with detergent (described below) followed by thorough rinsing with tapwater, deionized water, and undenatured absolute ethanol.

* The specification for the detergent used in cleaning the production equipment, including the product receivers is biodegradable, USDA/FDA approved for use in the food industry. Brand names are "Simple Green" and "Planisol-M".

* Absolute undenatured ethanol - USP grade, anhydrous, 200 proof.

* Deionized water - >16.7 megaohm-cm resistance.

* Alkali (KOH) - technical grade.

4. CHEMICAL COMPOSITION AND QUALITY ASSURANCE

The vegetable oils have been extensively analyzed by the Quality Assurance/Quality Control Project at the Charleston Laboratory for several categories of analysis: lipid classification, sterols, fatty acid oxidation products, organics, metals, moisture, and sensory attributes (Table 5-2a). The complete fatty acid compositions of the vegetable oils have been determined by capillary gas chromatography (Table 5-2b). The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

Peroxide value, iodine value, and moisture content were determined using automated titrators. Anisidine value, which is analyzed by standard IUPAC methods, is a measure of aldehydes. Tocopherols were analyzed to assure the concentrations added and to provide a measure of vitamin E activity.

The vegetable oils were analyzed for six potentially toxic metals: As, Se, Hg, Cd, Pb, and Ni. The products were also analyzed for eight macroelements which are of nutritional interest.

The fatty acid composition was determined by comparison of specific fatty acid relative retention time (RRT) with primary and secondary standards, by hydrogenation, argentation TLC, and GC/MS of their methyl esters. The method is described in detail in Appendix II.

Each of the vegetable oils was subjected to sensory analysis by a trained sensory evaluation panel for total intensity of odor (TIO), total intensity of flavor (TIF), and numerous different odor/flavor attributes. The values reported are based on an unstructured scale 15-cm in length, on which "0" represents "absent" and "15" represents "very strong" presence of a given attribute. The scores obtained on these products represent a rather mild odor/flavor profile.

TABLE 5-2a. CHEMICAL COMPOSITION AND QUALITY OF VEGETABLE OILS (PLACEBOS).

	VEGETABLE OILS		
ANALYSIS TYPE	OLIVE W86339W	SAFFLOWER Y86339Y	CORN V86339V
EPA, mg/g	<0.5	<0.5	<0.5
DHA, mg/g	<0.5	<0.5	<0.5
TOTAL n-3, mg/g	.8	1	9
FREE FATTY ACIDS, %	0.28	0.02	0.06
CHOLESTEROL, mg/g	<0.05	<0.05	<0.05
PEROXIDE VALUE, meq/kg	5.79	2.79	1.03
IODINE VALUE, g I_2/100g	90.8	139.9	126.7
ANISIDINE VALUE	5.88	21.4	5.25
ANTIOXIDANT CONTENT:			
a-TOCOPHEROL, mg/g	0.16	0.45	0.25
g-TOCOPHEROL, mg/g	0.10	<0.05	0.80
TBHQ, mg/g	*	*	*
MOISTURE, ug/g	192	205	178
PCBs, ug/g	<0.05	<0.05	<0.05
TOTAL DDT, ug/g	<0.05	<0.05	<0.05
TRACE METALS, ug/g:			
Arsenic	*	*	*
Cadmium	<0.10	<0.10	<0.10
Mercury	*	*	*
Lead	*	*	*
Selenium	*	*	*
MACRO ELEMENTS, ug/g:	*	*	*
Calcium	*	*	*
Chromium	*	*	*
Copper	<0.1	<0.1	<0.1
Iron	4.4	4.6	1.2
Potassium	*	*	*
Sodium	*	*	*
Tin	*	*	*
Zinc	<0.1	<0.1	<0.1
SENSORY ATTRIBUTES, 0-15, 15 MAX INTENSITY:			
ODOR:			
TIO**	2.81	2.24	2.4
BUTTERY	0.01	0.09	0
BEANY	0.1	0.31	0.12
RANCID	0	0	0
PAINTY	0.49	0.06	0.14
OXIDIZED	0.02	0.15	0.15
GRASSY	0.13	0.05	0.05
FISHY	0.06	0.1	0.3
BITTER	0	0	0
SWEET	0.52	0.51	0.91
FRUITY/MELON	0.83	0.01	0
BURNT	0.6	1.04	1.08

TABLE 5-2a. CONTINUED.

ANALYSIS TYPE	VEGETABLE OILS		
	OLIVE W86339W	SAFFLOWER Y86339Y	CORN V86339V
FLAVOR:			
TIF**	2.88	2.55	1.53
BUTTERY	0.52	0.49	0.12
BEANY	0.22	0.85	0.55
RANCID	0.01	0.34	0
PAINTY	0.51	0	0
OXIDIZED	0.47	0.23	0.21
GRASSY	0.06	0.04	0.04
FISHY	0	0	0.18
BITTER	0.09	0	0
SWEET	0.46	0.51	0.51
FRUITY/MELON	0.28	0.08	0.08
BURNT	0.23	0.35	0.46
HELLIGE No.:	7	4	4
BACTERIA			
MPN coliforms/g	<1	<1	<1
MPN fecal coliforms/g	<1	<1	<1
Salmonella cells/g	<1	<1	<1

* Not determined

** TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

TABLE 5-2b. FATTY ACID COMPOSITION (mg/g) OF VEGETABLE OILS (PLACEBOS).

FATTY ACID	VEGETABLE OILS		
	CORN L88333VV	OLIVE L88333WW	SAFFLOWER L88333YY
14:0	0.3	0.0	0.9
16:0	103.2	150.4	57.0
16:1n9	0.4	0.1	0.0
16:1n7	1.0	17.9	0.8
17:0	0.7	0.0	0.0
18:0	18.0	23.8	18.0
18:1n9	236.3	518.4	83.3
18:1n7	5.4	26.0	5.9
18:2n6	526.2	154.4	733.0
18:3n3	8.8	6.0	1.7
20:0	4.0	4.1	2.9
20:1	2.8	2.0	1.5
22:0	1.7	1.2	2.5
24:0	1.6	0.6	1.0
24:1	0.0	1.5	2.0

* Fatty acids were tentatively identified by comparison of their RRT values with those of primary and secondary standards and by GC/MS of their methyl esters.
** 0.0 = <0.05 mg/g

5. TECHNICAL SUPPORT

Technical support for the researchers by BTMP personnel includes a discussion of the experimental protocol. Input from the BTMP is focused on the development of the best protocol for the utilization of the test materials while maintaining their high quality. Each researcher approved for the requested amount of Biomedical Test Material (BTM) by the Fish Oil Test Material Distribution Committee (FOTMDC) receives a telephone call soon after receipt of the approval letter. The initial conversation covers a diversity of topics depending upon the research need and protocol. The information in the application is verified in terms of research objective, experimental protocol, and the mode of administration. Information on the availability, composition, storage, and stability data of the BTM is provided. The amount of technical assistance provided to each researcher varies, depending upon their specific needs. Some researchers have significant experience in the n-3 research field and require less assistance than those just entering the n-3 arena. Technical support is provided in the forms listed below:

a. Suitability of type and quantity of requested BTM for proposed research protocol.

b. Literature information obtained from the Fish Oil Bibliography.

c. Analytical data, including the Quality Assurance data.

d. Antioxidants present in the BTM and balancing of placebo test materials including analysis of placebo oils.

e. Use of control/placebo treatments in experimental designs; flavoring/masking of placebo treatments in double blind trials.

f. Customized packaging and scheduling of shipments of BTM.

Additional information is provided the researcher in the form of printed materials and includes:

g. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

h. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules" (Appendix 4).

i. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil" (Appendix 5).

j. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids.

k. Technical information on the proper storage of each of the test materials to prevent their oxidative deterioration and to ensure maintenance of the high quality of the delivered test material.

l. Technical information is provided in support of IND (Investigative New Drug) applications: The use of BTMs in human studies requires the acquisition of an IND number from the Food and Drug Administration. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. This Drug Master File (DMF) containing technical information on the chemical composition, processing, and handling of the specific

test materials serves to expedite IND requests for researchers approved for using the BTM in their studies. The NIH/Fish Oil Test Material Program (FOTMP) office submits the names of approved researchers planning human studies to the FDA to authorize access to the appropriate DMF.

m. NOAA Technical Memorandum NMFS-SEFC-234; "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1).

B. ENCAPSULATION of VEGETABLE OILS - First Encapsulation.

Researchers involved in human studies usually use fish oil and the appropriate placebo oil in the soft-gelatin capsule form. On rare occasion, capsules are utilized to introduce test materials to primates. They are also ideal `containers' for storage of secondary analytical standards.

1. SOFT GEL ENCAPSULATION PROCESS INFORMATION

a. Introduction

Three vegetable oils were ordered from Columbus Foods, Chicago, IL, for encapsulation as placebos by the Chase Chemical Co., Newark, NJ. Grade "A" olive oil, food grade corn oil, and safflower oils were packaged in one gallon plastic containers. The oils were shipped directly to the encapsulator by the supplier.

b. Process Description

All of the oils were encapsulated as No. 20 oblongs containing one g oil/capsule in an "air-free" system under "hospital" grade N_2, using conventional rotary die equipment. Corn oil was the first of the oils to be encapsulated, followed by olive oil and then safflower oil. This follows the industry practice of encapsulating the oil of lowest specific gravity first, and encapsulating all placebo oils before the actual test material. The gel formulation contained titanium dioxide as an opacifier to conceal the color of the oils contained within the capsules. After washing and drying, as per industry practices, the capsules were packaged.

2. PACKAGING MATERIALS SPECIFICATIONS

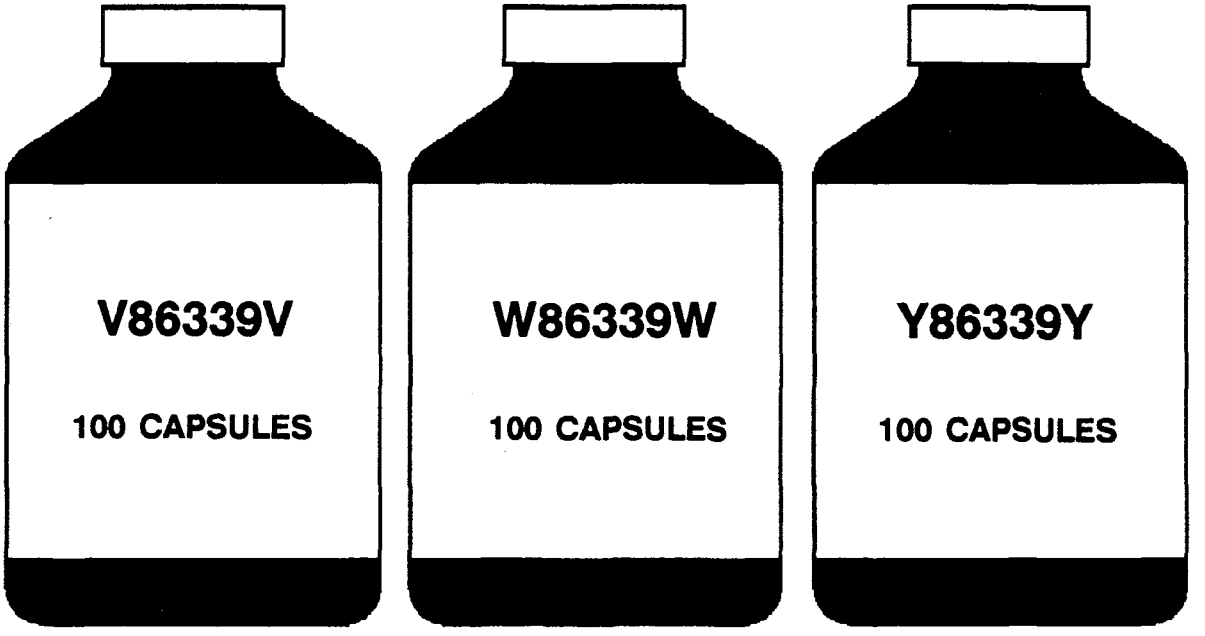
a. Containers

The capsules were packaged in brown glass bottles, 100 capsules (1000 mg/cap)/bottle, with screw-cap lids and external tamper-proof seals. The bottles were packaged 24 to a shelf-pack, three shelf-packs to a case. The individual shelf-packs were labeled with the `lot number' pertaining to the product contained within, while the outer carton was labeled corn oil, olive oil, or safflower oil as appropriate to the contents.

b. **Labels**

An example of the label used on bottles of encapsulated vegetable oils appears in Figure 5-1 and contains the following information: LOT XXXXXXXX, 100 Capsules (1,000 mg). Additional labels are sent with these bottles to all users; a cover letter explains that it is the investigator's responsibility to use the additional labels in their studies. The additional label contains the following information: BIOMEDICAL TEST MATERIAL, 100 Capsules (1000 mg), Keep refrigerated, "CAUTION: NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE".

FIGURE 5-1. EXAMPLE LABELS FOR BOTTLES OF SOFTGEL CAPSULES OF VEGETABLE OILS.



3. STORAGE STABILITY

Storage stability of three encapsulated vegetable oil placebos, olive, corn, and safflower oils, stored in brown glass bottles at 5°C was determined by analysis of fatty acid composition, free fatty acids, and peroxide value at 0 time and 12 months after encapsulation. The results of this study are presented in Table 5-3 and in Appendix 4, "Storage Stability of Steam-deodorized Menhaden Oil in Soft Gelatin Capsules". No significant changes in oil quality were observed.

C. ENCAPSULATION of VEGETABLE OILS - Subsequent Encapsulations.

1. SOFT GEL ENCAPSULATION PROCESS INFORMATION

a. Introduction

The rotary die process, invented by R.P. Scherer in 1933, is a continuous process for producing soft gelatin capsules. The rotary die process reduced manufacturing losses to a negligible figure and content variation to ± 1-3%. Capsules are manufactured and partially dried in the following three continuous steps:

* Two gelatin ribbons are prepared, automatically and continuously, and fed, with the fill material, to the encapsulating mechanism.
* The capsules are simultaneously and continuously filled, with the force of the injected fill material causing the gelatin to expand into the die pockets to form the shape of the product, hermetically sealed and automatically cut between two rotary dies.
* The formed capsules are automatically conveyed to and through a solvent wash unit and partially dried in a forced-air tunnel.

The gelatin contains approximately 30 percent water and is heated to a temperature of 37-40°C. The physical characteristics of gelatin are related to the (1) "bloom", a measure of the cohesive strength of the cross-linking, which occurs between gelatin molecules, and is proportional to the molecular weight of the gelatin, and (2) viscosity, a measure of the molecular chain length which determines the manufacturing characteristics of the gelatin film.

After the capsules are formed and washed with solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5% of the water. The product is then subjected to drying in a forced air oven (24% R.H., 70°F) for 16 hours which produces a capsule with 6-10% water content at equilibrium.

b. Process Description

Vegetable oils are encapsulated at General Nutrition Corporation (GNC), Greenville, SC with a fill weight of 1,000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. The uniformity of dosage units utilized conforms with USP XXI specifications (pp. 1277-1278). A NMFS BTM program representative inspects and provides oversight at encapsulation facilities during encapsulation. It appears that good manufacturing practices are being utilized at the facility during encapsulation of the test materials. Several modifications of the encapsulation process are performed to minimize contact of the BTM with oxygen and maintain their high quality. The following precautions are taken during encapsulation of the test materials.

* Initial transfer of the fill material from the shipping drum into the process container, routinely consists of the drum being lifted and the contents emptied, causing significant mixing of air into the product. This procedure is altered so that the BTM's are transferred via a transfer line using nitrogen pressure to the process container flushed with nitrogen. The container is blanketed with nitrogen and closed with an air-tight cover during mixing prior to encapsulation.
* During the packaging of the test materials, nitrogen flushing of the bottled capsules is incorporated at the bottle slow down point (3 sec flush time), prior to capping.
* Brown, polyethylene bottles are used to provide the best protection for the test materials while reducing shipping weight of the product.

After encapsulation, the bottled capsules are flushed with nitrogen prior to application of the inner heat seal. Labels are applied on line and are described in Section V-B.2.b of this document. The capsules are stored at 5°C at the Charleston Laboratory.

c. Chemical Composition of Encapsulating Materials

The components used by GNC in formulating the capsule material are gelatin, water, and glycerin. In addition the capsules are rinsed with perchloroethylene. Information regarding the composition of these components is presented below.

* gelatin - Information is provided in Figure 5-2.
* glycerin - Information is provided in Figure 5-3.
* perchloroethylene - meets USP specification requirements for internal use in humans, as stated by the encapsulator.

d. Quality Assurance of Encapsulating Materials

* gelatin - Information is provided in Figure 5-2.

* glycerin - Information is provided in Figure 5-3.

* perchloroethylene residue - The test method consisted of perchloroethylene residue headspace analysis performed on capsules at different stages in the process: directly off the machine, at the end of the dryer prior to the oven, and after 16 and 32 hours of oven drying. Based on a headspace analysis method, the capsules contain no detectable level of perchloroethylene residue after drying (equal to or below the detection limit of "background noise" which is approximately 0.4-0.5 ppm).

* bacterial count on gelatin capsule after curing - After case hardening, the capsules are subjected to microbiological analyses. The encapsulator performs a pathogen screen and standard plate count. All lots have been accepted with a negative pathogenic screening and a standard plate count of <10 CFU/gm. In addition, the NMFS Charleston Laboratory analyzes the gelatin of the encapsulated oils for coliforms and *Salmonella* using the procedures presented in the QA Methods Manual (Appendix 2).

FIGURE 5-2. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Hide Gelatin Special Blend

RM Listing Designation: Hide Gelatin (P.C.) Blend **RM Code:8626**

Specifications:

Description	: Sheets, flakes, shreds or a coarse to fine powder; faintly yellow or amber in color; the color varying in depth according to particular size; slight characteristic bouillon-like odor in solution
Identification	: Positive by visual inspection and compare to type
Assay/Gel Strength	: 150.0 to 165.0 Bloom
Sieve Analysis	: Not less than 100% through a 16 U.S. Standard Sieve Not less than 1% through a 100 U.S. Standard Sieve
LOD	: Not more than 13%
Arsenic	: Not more than 0.8ppm
Heavy Metals	: Not more than 50 ppm (0.005%) (as Pb)
pH	: 5.8 - 6.2
Residue on Ignition	: Not more than 2.0%
Solubility	: Insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, acetic acid, and hot mixtures of glycerin and water; insoluble in alcohol, chloroform, ether, and fixed and volatile oils.
Viscosity	: 41 $\pm$ 3 mps at 60 degrees C
Dioxide	: Not more than 0.15%
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Pathogens : Negative : Standard Plate Count: Less than 10,000 CFU/gm : Mold & Yeast : Less than 5000 CFU/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot : received. The C of A may be used in place of any of all : testing required above to verify the material meets : specifications.
References	: Vendor Protocol

FIGURE 5-3. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Glycerin - Glycerol 92.09

RM Listing Designation: Glycerin, Natural 99.5% **RM Code:8194**

Specifications:

Description	: Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic.
Identification	: Positive by IR comparison
*Assay	: NLT 95.0% - NMT 101.0%
*Specific Gravity	: NLT 1.249
Solubility	: Miscible with water, alcohol, and methanol
Insoluble	: Chloroform, ether, and fixed & volatile oils
Color	: Clear, colorless liquid / GNC Gardner Scale 1
*Arsenic	: 1.5 ppm
*Heavy Metals	: Limit is 5 ppm
Loss on Ignition	:
Saponification Value:	
*Chloride	: 0.001%
*Sulfate	: NMT 0.002%
*Fatty Acids and Esters:	NMT 1 ml of 0.5 sodium hydroxide is consumed
*Chlorinated compounds:	(0.003% of Cl)
Iodine Value	:
Unsaponifiable Matter (%)	:
Viscosity	: 995 Cps at 25 C, Brookfield apparatus RVT
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Coliforms : Less than 1,000 MPN/gm : Standard Plate Count: Less than 5000 CFU/gm : Mold & Yeast : Less than 500 CFU/gm : Fecal Coliforms : Less than 100 MPN/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot received. The product code number should also be shown on each container and certificate of analysis forwarded to Quality Control.
References	: U.S. Pharmacopeia XXI, p. 464

* Designates official USP XXI specification

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

The containers used for bottling capsules of ethyl esters of vegetable oils are amber high density linear polyethylene bottles as described in Figure 5-4. These specifications were provided by the encapsulator, GNC.

FIGURE 5-4. GENERAL SPECIFICATIONS FOR PLASTIC BOTTLES.

REVISED: JUNE 14, 1985

MEETS USP III CONTAINER SPECIFICATIONS PAGES 1238-1240.

Amber Plastic Bottles

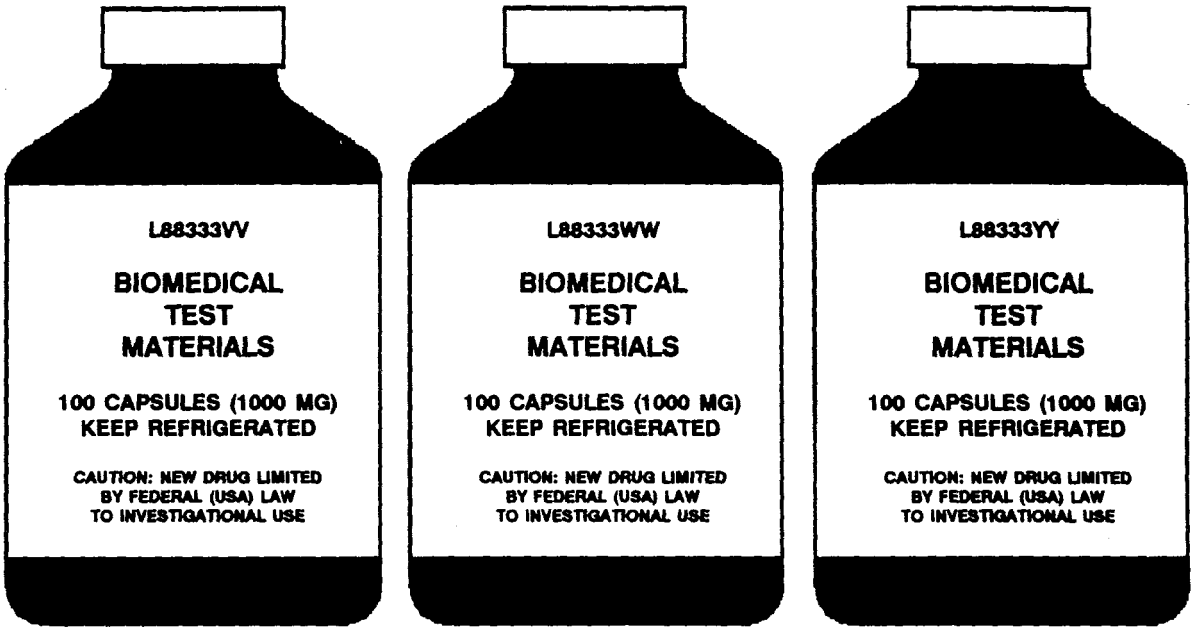
a. Resin - high density, linear polyethylene
b. Pigment - Iron Oxide
c. Mold Release Compound - Zinc Stearate 0.15 percent
d. Flame Treated
e. Style M Necks

MINIMUM WALL THICKNESS	
<500 cc	0.025"
500 cc	0.035"
625 cc	0.035"
750 cc	0.038"
950 cc	0.040"
1300 cc	0.047"

b. Labels

An example label used on bottles of capsules of vegetable oils appears in Figure 5-5 and contains the following information: BIOMEDICAL TEST MATERIALS, LOT XXXXXXXX, 100 Capsules (1000 mg), Keep Refrigerated, "Caution: New Drug Limited by Federal (USA) Law to Investigational Use".

FIGURE 5-5. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF VEGETABLE OILS.



3. STORAGE STABILITY of SOFTGEL ENCAPSULATED VEGETABLE OILS

Storage stability of vegetable oils in soft gelatin capsules at 5°C is currently being studied. The data are summarized in Table 5-3.

TABLE 5-3. STORAGE STABILITY OF SOFTGEL CAPSULES OF VEGETABLE OILS.

	OLIVE		CORN		SAFFLOWER	
TIME, WEEKS	0	52	0	52	0	52
EPA, mg/g	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DHA, mg/g	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
TOTAL n-3, mg/g	7	7.6	9	9	1	1
FREE FATTY ACIDS, %	0.29	0.16	0.06	0.13	0.02	0.02
PEROXIDE VALUE	5.97	5.13	1.03	1.22	2.79	2.75
IODINE VALUE	90.8	*	126.7	*	139.9	*

* Not determined.

D. BULK PACKAGING of VEGETABLE OILS

Researchers conducting animal experimentation primarily utilize fish oils in the bulk form. Therefore placebo vegetable oils are also required in bulk. Bulk packaging of placebo vegetable oils is not conducted at the Charleston facility.

The vegetable oils are ordered by the researcher and analyzed by the Charleston laboratory for tocopherol content. The researcher is advised of the tocopherol content and given instructions on the balancing of their placebo oil with the Charleston Laboratory fish oil test material. Analytical results of final concentration of antioxidant and tocopherol levels in the vegetable oil may also be verified upon submission of a sample after addition of antioxidant and tocopherols. For these analyses, the investigator is requested to send 5ml of the oil in an amber glass or plastic container.

SECTION VI. PROCESSING/CHEMICAL COMPOSITION DATA FOR OMEGA-3 FATTY ACID ETHYL ESTERS.

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A. GENERAL INFORMATION

1. STARTING MATERIAL SPECIFICATIONS:

a. Vacuum deodorized fish oil (VDFO)

Vacuum deodorized fish oil (VDFO) is produced from partially refined menhaden oil which is obtained from the small oily menhaden, ***Brevoortia sp.*** VDFO is pale yellow in color and has a characteristic odor and flavor. Details of the process by which menhaden oil is refined are provided in Section IV of this Drug Master File. The quality specifications for vacuum deodorized fish oil produced at the Charleston Laboratory are given in Table 6-1.

TABLE 6-1. QUALITY SPECIFICATIONS FOR VACUUM DEODORIZED FISH OIL.

TRIGLYCERIDES, %	>92
EPA, mg/g	>120
DHA, mg/g	>75
FREE FATTY ACIDS, %	<0.2
TRANS ACIDS, %	<5
CHOLESTEROL, mg/g	<5.0
PEROXIDE VALUE, meq/kg	<5.0
IODINE VALUE, g I_2/100g	>160
ANISIDINE VALUE	<50
a-TOCOPHEROL, mg/g	0.5-5.0
g-TOCOPHEROL, mg/g	0.5-5.0
TBHQ, mg/g	0.1-0.2
MOISTURE, ug/g	<500
PCBs, ug/g	<0.5
TOTAL DDT, ug/g	<0.5
TRACE METALS, ug/g:	
Arsenic	<1.0
Cadmium	<1.0
Lead	<1.0
Mercury	<1.0
Selenium	<1.0
SENSORY ATTRIBUTES:	
ODOR (TIO)	<6.0
FLAVOR (TIF)	<6.0
OTHER:	
SPECIFIC GRAVITY	0.93
SOLIDIFICATION RANGE	**
SAPONIFICATION VALUE	191-200
UNSAPONIFIABLE MATTER	<1.3%

* TIO= Total intensity of odor; TIF= Total intensity of flavor; using a 15 cm scale.

** VDFO is a liquid at 5°C or higher.

2. PROCESSING INFORMATION:

a. Production of n-3 Concentrates and Purified Fractions

The feedstock for production of concentrates and purified fractions is vacuum deodorized fish oil, described above in `Starting Material Specifications', and in Section IV of this Drug Master File. The first step in production of the n-3 concentrate is the preparation of fatty acid ethyl esters using absolute undenatured ethanol and sodium ethoxide synthesized in the production plant from metallic sodium and absolute ethanol. Two glass reactors (72 L) are used to prepare 80 Kg of ethyl esters (40 Kg in each reactor) from 80 Kg of deodorized oil in a N_2 atmosphere. After reaction is complete, the solution is allowed to stand until it separates into two phases. The lower alcoholic glycerol phase is drained off and the upper phase, consisting of the esters and some alcohol, is pumped into a 100 L glass separatory funnel, under N_2, where the esters are washed several times with deionized water. Following the final wash, the esters are left overnight in the funnels to allow complete separation of the water from the esters.

Prior to transferring the esters to the crystallizer, any visible water in the separatory funnel is drained. The esters are then pumped into the crystallizer which contains a hot (80°C) solution of urea dissolved in 95% ethanol, under N_2. As soon as the esters have dissolved completely (about 20 min), the solution is cooled by circulating cold water through the jacket of the crystallizer. The speed of the scraped-wall mixer of the crystallizer is reduced to its lowest setting and the mixture is cooled to 5°C and held overnight.

The liquid in the crystallizer, containing the more unsaturated ethyl esters is first syphoned from the crystallizer, and later pumped from the bottom of the crystallizer, to a scraped-wall film evaporator where most of the alcohol is distilled from the product. The product is then pumped to a 100 L separatory funnel and washed, under N_2, first with dilute HCl and then with deionized water. Finally, the crude concentrates are pumped into 10 L Schott bottles, blanketed well with N_2, and stored overnight at 5°C.

The crude concentrates are distilled in a glass/Teflon two-stage wiped film molecular still. In the first stage, any residual ethanol is distilled along with most of the contaminating 16 carbon polyunsaturates (which are not n-3 esters). The n-3 esters, primarily 18:4n3, 20:5n3 and 22:6n3, are distilled in the second stage, leaving color bodies, polymers, and most of the cholesterol as an undistilled residue.

This purified concentrate serves as feedstock for further purification of 20:5n3 and 22:6n3 by supercritical fluid CO_2 (SCF-CO_2) fractionation. At temperatures above 31.1°C and pressures above 1070 psi, CO_2 behaves as a fluid with substantial power as a solvent. At pressures of 1900-2500 psi, CO_2 is passed through a charge of esters (200 g), dissolving them and carrying them through a stainless steel column packed with Propak, a patented stainless steel column packing material. The dissolved esters pass through seven heated zones (43°C in the middle of the column to 80°C at the top). As the temperature is increased, 20:5n3 and 22:6n3 become less soluble in the liquid than the more soluble shorter chained esters.

As 20:5 and 22:6 drain back down through the column packing, a reflux is established which improves the separation of 20:5 and 22:6 from the other components of the mixture. Five fractions of the column effluent are collected in 1 gal stainless steel pressure vessels at atmospheric pressure. The fractions are protected from oxidation by gaseous CO_2 in the vessels. The last three fractions, together, contain 82% of the 20:5 and 99% of the 22:6 present in the feedstock.

Fractions collected from the SCF-CO_2 fractionator are passed through a reverse phase column installed in a high performance liquid chromatograph, using 80% aqueous ethanol as a solvent, to produce 20:5 and 22:6 in purities of 99%. The solvent is removed using an all glass/Teflon film evaporator.

b. Specifications for Chemicals, Solvents and Additives Used in the Process:

* Tocopherols and antioxidant TBHQ - The tocopherols (alpha- and gamma-tocopherol) and the antioxidant tertiary butylhydroquinone (TBHQ) are added to the vacuum deodorized fish oil at the Charleston Laboratory so that the product, n-3 ethyl ester concentrate, has approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, 0.02% TBHQ. TBHQ is added as a mixture containing the components listed below. The tocopherols and the TBHQ are added to increase the stability and shelf life of the fish oil. Tocopherols and TBHQ contained in the ester concentrates are those added in the initial vacuum-deodorizing process: Vitamin E 5-67, Tenox GT-1, and Tenox 20A (all products are from Eastman Chemical Co., Rochester, NY). These products have been analyzed at the Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils. The products contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
Tenox GT1	Mixed tocopherols (alpha, gamma, delta) 50% Vegetable oil 50%
Vitamin E 5-67	672 mg/g d-alpha-tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha).

* Absolute undenatured ethanol - USP grade, anhydrous, 200 proof.

* Metallic sodium - ACS grade packed under vacuum.

* Gaseous nitrogen - FDA approved and <5 ppm O_2.

* Hydrochloric acid - ACS grade.

* Deionized water - >16.7 megaohm-cm resistance.

* Gaseous CO_2 - USP grade, not less than 99.0% CO_2 by volume.

* Aqueous alcoholic solutions - undenatured, USP grade, anhydrous, 200 proof ethanol diluted appropriately with deionized water.

* Alkali (KOH) - technical grade.

3. EQUIPMENT MAINTENANCE:

Specific cleaning methods, including frequency, are described in the Production Methods and Safety Manual (Appendix 1). All major production equipment is cleaned with materials described above (undenatured alcohol, deionized water, and alcoholic alkali). All glassware and stainless-steel product receivers are cleaned with detergent (described below) followed by thorough rinsing with tapwater, deionized water, and undenatured absolute ethanol.

* The specification for the detergent used in cleaning the production equipment, including the product receivers is biodegradable, USDA/FDA approved for use in the food industry. Brand names are "Simple Green" and "Planisol-M".

4. CHEMICAL COMPOSITION AND QUALITY ASSURANCE:

a. Specifications for n-3 Ethyl Ester Concentrates of Menhaden Oil

Quality assurance specifications for n-3 ethyl ester concentrates produced at the Charleston Laboratory are listed in Table 6-2. All test materials produced at the laboratory must be certified to meet these specifications before shipment for researchers. To assure potency of the n-3 concentrate, minimum concentrations of 400 mg/g EPA and 200 mg/g DHA are specified, as well as a total n-3 content of no less than 700 mg/g. A number of specifications are set to assure lipid quality and stability: peroxide value, anisidine value, free fatty acid content, moisture, trans fatty acids, sensory analysis, and antioxidant content. Other specifications delimit acceptable levels of contaminants in the product: cholesterol, metals, PCBs and pesticides, and residual urea. All limits established for contaminants are well below established FDA limits in foods.

b. Specifications for n-3 Ethyl Esters of EPA and DHA

Quality assurance specifications for the ethyl esters of the purified n-3 fatty acids, EPA and DHA, produced at the Charleston Laboratory are listed in Table 6-2. All test materials produced at the laboratory must be certified to meet these specifications before shipment for researchers. To assure potency of the test material, minimum and maximum concentrations of EPA and DHA are specified respectively, as well as total n-3 content. A number of specifications are set to assure lipid quality and stability: peroxide value, free fatty acid content, moisture, trans fatty acids, sensory analysis, and antioxidant content. Other specifications delimit acceptable levels of contaminants in the product: cholesterol, metals, PCBs and pesticides, and residual alcohol. All limits established for contaminants are well below established FDA limits in foods.

TABLE 6-2. QUALITY SPECIFICATIONS FOR FISH OIL DERIVED n-3 ETHYL ESTERS TO BE SHIPPED FROM CHARLESTON LABORATORY.

	TEST MATERIAL		
ANALYSIS TYPE	n-3 CONC	EPA	DHA
ESTERS, %	>90	>95	>95
EPA, mg/g	>400	>900	<50
DHA, mg/g	>200	<50	>900
TOTAL n-3, mg/g	>700	>950	>950
FREE FATTY ACIDS, %	<0.2	<0.2	<0.2
TRANS ACIDS, %	<5	<5	<5
CHOLESTEROL, mg/g	<5.0	<0.1	<0.1
PEROXIDE VALUE, meq/kg	<10.0	<5.0	<5.0
IODINE VALUE, g I_2/100g	>320	*	*
ANISIDINE VALUE	<80	*	*
ANTIOXIDANT CONTENT:			
a-TOCOPHEROL, mg/g	0.5-5.0	**	**
g-TOCOPHEROL, mg/g	0.5-5.0	**	**
TBHQ, mg/g	0.1-0.2	**	**
MOISTURE, ug/g	<500	<500	<500
RESIDUAL UREA, ug/g	<20	<20	<20
PCB, ug/g	<0.5	<0.5	<0.5
TOTAL DDT, ug/g	<0.5	<0.5	<0.5
TRACE METALS, ug/g:			
Arsenic	<1.0	<1.0	<1.0
Cadmium	<1.0	<1.0	<1.0
Lead	<1.0	<1.0	<1.0
Mercury	<1.0	<1.0	<1.0
Selenium	<1.0	<1.0	<1.0
SENSORY ATTRIBUTES:			
ODOR (TIO)	<6.0	*	*
FLAVOR (TIF)	<6.0	*	*
OTHER:			
SPECIFIC GRAVITY	0.89	**	**
SOLIDIFICATION RANGE	***	***	***

* Not applicable
** Not enough material to conduct these analyses routinely
*** Esters are a liquid at 5° C or higher.

c. Chemical Composition of n-3 Concentrates

The n-3 ester concentrates have been extensively analyzed by the Quality Assurance/Quality Control Project at the Charleston Laboratory for several categories of analysis: lipid classification, sterols, fatty acid oxidation products, organics, metals, moisture, and sensory attributes (Table 6-3a). The complete fatty acid composition of the ester concentrate as well as the purified fractions of EPA and DHA have been determined by capillary gas chromatography (Tables 6-3b). The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

Peroxide value, iodine value, and moisture content were determined using automated titrators. Anisidine value, which is analyzed by standard IUPAC methods, is a measure of aldehydes. Tocopherols were analyzed to assure the concentrations added and to provide a measure of vitamin E activity. Urea was analyzed to determine the presence of trace amounts of urea remaining from the urea adduction step in the production process.

The esters were analyzed for six potentially toxic metals: As, Se, Hg, Cd, Pb, and Ni. The products were also analyzed for eight macroelements which are either of nutritional interest or may potentially be picked up during processing.

The fatty acid composition was determined by comparison of specific fatty acid relative retention time (RRT) with primary and secondary standards, by hydrogenation, argentation TLC, and GC/MS of their methyl esters. The method is described in detail in Appendix 2.

The products were subjected to sensory analysis by a trained sensory evaluation panel for total intensity of odor (TIO), total intensity of flavor (TIF), and eleven different odor/flavor attributes. The values reported are based on an unstructured scale 15-cm in length, on which "0" represents "absent" and "15" represents "very strong" presence of a given attribute. The scores obtained on these products represent a rather mild odor/flavor profile.

TABLE 6-3a. CHEMICAL COMPOSITION OF n-3 ETHYL ESTERS.

ANALYSIS TYPE	TEST MATERIAL n-3 CONC L88333BF	EPA	DHA
ESTERS, %	92	95	95
EPA, mg/g	414	914	2
DHA, mg/g	240	0	909
TOTAL n-3, mg/g	783	919	931
FREE FATTY ACIDS, %	0.23	*	0.07
TRANS FATTY ACIDS, %	<5	*	*
CHOLESTEROL, mg/g	2.0	<0.05	<0.05
PEROXIDE VALUE, meq/kg	1.92	1.61	*
IODINE VALUE, g I_2/100g	370	*	*
ANISIDINE VALUE	39.6	*	*
ANTIOXIDANT CONTENT:			
a-TOCOPHEROL, mg/g	1.5	0.37	<0.05
g-TOCOPHEROL, mg/g	2.1	1.38	<0.05
TBHQ, mg/g	*	*	*
UREA, ug/g	*	*	*
MOISTURE, ug/g	465	1559	*
PCBs, ug/g	*	*	*
TOTAL DDT, ug/g	*	*	*
SENSORY ATTRIBUTES,			
0-15, 15 MAX INTENSITY:			
ODOR:			
TIO	4.96	2.74	3.53
BUTTERY	0	0	0
BEANY	0	0.08	0
RANCID	0	0	0.4
PAINTY	1.06	0.16	0
OXIDIZED	0	0	0
GRASSY	0.41	0.66	1.85
FISHY	0	0.24	0.77
BITTER	0.89	0	0
SWEET	0	0.08	0
FRUITY/MELON	1.46	0.72	1.47
BURNT	0	0	0
SOLVENT	1.54	0.74	1.10
SOAPY	1.63	0	0
VARNISH	0	0.48	0

TABLE 6-3a. CONTINUED.

	TEST MATERIAL		
ANALYSIS TYPE	n-3 CONC	EPA	DHA
FLAVOR:			
TIF	5.31	3.00	4.37
BUTTERY	0	0	0
BEANY	0	0	1.33
RANCID	0	0.26	0
PAINTY	1.37	0	0
OXIDIZED	0.37	0	0
GRASSY	0.37	0.88	1.1
FISHY	0.28	0.8	1.03
BITTER	2.04	0.16	1.3
SWEET	0	0.64	0
FRUITY/MELON	1.26	1.14	0.53
BURNT	0	0	0
SOLVENT	2.96	0.5	0.73
SOAPY	2.44	0	0
VARNISH	0	0.5	0

* Not determined.

TABLE 6-3b. FATTY ACID COMPOSITION (mg/g) OF n-3 ETHYL ESTERS*.

	TEST MATERIAL		
ANALYSIS TYPE	n-3 CONC L88333BF	EPA	DHA
16:2n7	0.0**	0.0	0.0
16:2n6	0.0	0.0	0.0
16:2n4	6.4	0.0	0.0
18:2n9	0.0	0.0	0.0
18:2n7	0.0	0.0	0.0
18:2n6	0.9	0.5	0.0
18:2n4	0.5	0.0	0.0
20:2n6	1.1	0.0	0.0
TOTAL DIENES	8.9	0.5	0.0
16:3n4	35.9	0.0	0.0
16:3n3	5.1	0.0	0.0
16:4n1	23.2	0.5	0.0
18:3n6	5.5	8.8	0.0
18:3n4	6.3	0.0	0.0
18:3n3	2.1	1.9	0.0
18:4n3	68.0	2.0	0.0
18:4n1	4.7	0.0	0.0
20:3n6	2.2	0.0	0.3
20:4n6	22.1	0.0	7.3
20:3n3	2.1	0.0	0.0
20:4n3	7.2	0.5	2.2
20:5n3	413.5	913.8	2.0
21:5n3	14.8	0.4	9.6
22:4n6	0.6	0.0	0.0
22:5n6	6.1	0.0	0.0
22:5n3	20.6	0.0	7.4
22:6n3	236.0	0.0	909.4
TOTAL n-3	769.4	918.5	930.6
TOTAL n-6	38.5	8.8	7.5
n-3/n-6	20.0	104.1	123.5
TOTAL PUFA	884.9	944.7	948.8

* Ethyl esters were tentatively identified by comparison of their RRT with those of primary and secondary standards, by hydrogenation, argentation TLC, and GC/MSD of the esters.
** 0.0 = <0.05 mg/g

5. TECHNICAL SUPPORT:

Technical support for the researchers by BTMP personnel includes a discussion of the experimental protocol; input from the BTMP is focused on the development of the best protocol for the utilization of the test materials while maintaining their high quality. Each researcher approved for the requested amount of Biomedical Test Material (BTM) by the Fish Oil Test Material Distribution Committee (FOTMDC) receives a telephone call soon after receipt of the approval letter. The initial conversation covers a diversity of topics depending upon the research need and protocol. The information in the application is verified in terms of research objective, experimental protocol, and the mode of administration. Information on the availability, composition, storage, and stability data of the BTM is provided. The amount of technical assistance provided to each researcher varies depending upon their specific needs. Some researchers have significant experience in the n-3 research field and require less assistance than those just entering the n-3 arena. Technical support may be provided in the forms listed below:

a. Suitability of type and quantity of requested BTM for proposed research protocol.

b. Literature information obtained from the Fish Oil Bibliography.

c. Analytical data, including the Quality Assurance data.

d. Antioxidants contained in the BTM and balancing of placebo test materials including analysis of placebo oils.

e. Use of control/placebo treatments in experimental designs; flavoring/masking of placebo treatments in double blind trials.

f. Customized packaging and scheduling of shipments of BTM.

Additional information is provided the researcher in the form of printed materials and includes:

g. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

h. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules" (Appendix 4).

i. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking sensory Attributes of Fish Oil" (Appendix 5).

j. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids.

k. Technical information on the proper storage of each of the test materials to prevent their oxidative deterioration and to ensure maintenance of the high quality of the delivered test material.

l. Technical information is provided in support of IND (Investigative New Drug) applications: The use of BTM in human studies requires the acquisition of an IND number from the Food and Drug Administration. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. This Drug Master File (DMF) containing technical information on the

chemical composition, processing, and handling of the specific test materials serves to expedite IND requests for researchers approved for using the BTM in their studies. The NIH/Fish Oil Test Material Program (FOTMP) office submits the names of approved researchers planning human studies to the FDA to authorize access to the appropriate DMF.

m. NOAA Technical Memorandum NMFS-SEFC-234; "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1).

B. ENCAPSULATION OF n-3 ETHYL ESTER CONCENTRATE

Researchers involved in human studies usually use ethyl esters of fish oil in the soft-gelatin capsule form. On rare occasion, capsules are utilized to introduce test materials to primates. They are also ideal `containers' for storage of secondary analytical standards.

1. SOFT GELATIN ENCAPSULATION PROCESS INFORMATION

a. Introduction

The rotary die process, invented by R.P. Scherer in 1933 is a continuous process for producing soft gelatin capsules. The rotary die process reduced manufacturing losses to a negligible figure and content variation to $\pm$ 1-3%. Capsules are manufactured and partially dried in the following three continuous steps:

* Two gelatin ribbons are prepared, automatically and continuously, and fed with the product to the encapsulating mechanism.
* The capsules are simultaneously and continuously filled, with the force of the injected product causing the gelatin to expand into the die pockets to form the shape of the product, hermetically sealed and automatically cut between two rotary dies.
* The formed capsules are automatically conveyed to and through a solvent wash unit and partially dried in a forced-air tunnel.

The gelatin contains approximately 30 percent water and is heated to a temperature of 37-40°C. The physical characteristics of gelatin are related to the (1) "bloom", a measure of the cohesive strength of the cross-linking, which occurs between gelatin molecules, and is proportional to the molecular weight of the gelatin, and (2) viscosity, a measure of the molecular chain length which determines the manufacturing characteristics of the gelatin film.

After the capsules are formed and washed with solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5% of the water. The product is then subjected to drying in a forced air oven (24% R.H., 70°F) for 16 hours which produces a capsule with 6-10% water content at equilibrium.

b. Process Description

Omega-3 ethyl ester concentrate is encapsulated at General Nutrition Corporation (GNC), Greenville, SC with a fill weight of 1,000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. The uniformity of dosage units utilized conforms with USP XXI specifications (pp. 1277-1278). A NMFS BTM program representative inspects and provides oversight at encapsulation facilities during encapsulation of the n-3 ethyl ester concentrate. It appears that good manufacturing practices are being utilized at the facility during encapsulation of the n-3 ethyl ester concentrate. Several modifications of the encapsulation process are performed to minimize the contact of the BTM with oxygen and maintain the high quality of the BTM. The following precautions are taken during encapsulation of the test materials.

* Initial transfer of materials from their container into the process vat routinely consists of the container being lifted and the contents emptied, causing significant mixing of air into the product. This procedure is altered so that the contents are transferred via a transfer line using nitrogen pressure to a container flushed with nitrogen. The process vat is blanketed with nitrogen and closed with an air-tight cover during mixing and prior to encapsulation.
* During the packaging of the n-3 ethyl ester concentrate, nitrogen flushing of the bottled capsules is incorporated at the bottle slow down point (3 sec flush time), prior to capping.
* Brown, polyethylene bottles are used to provide the best protection for the test materials while facilitating shipping of the product.

These capsules are maintained in a nitrogen atmosphere in temperature controlled containers prior to bottling. After bottling, the capsules are flushed with nitrogen prior to application of the inner heat seal. Labels are applied on line and are described in Section VI-B.2.b of this document. The capsules are stored at 5°C at the Charleston Laboratory.

c. Chemical Composition of Materials Utilized in the Encapsulation Process

The components used by GNC in formulating the capsule material are gelatin, water, and glycerin. Information regarding the composition of these components is presented below.

* gelatin - Information is provided in Figure 6-1.
* glycerin - Information is provided in Figure 6-2.
* perchloroethylene - meets USP specification requirements for internal use in humans, as stated by the encapsulator, GNC.

d. **Quality Assurance of Encapsulating Materials**

* gelatin - Information is provided in Figure 6-1.

* glycerin - Information is provided in Figure 6-2.

* perchloroethylene wash - The test method consisted of headspace/gas chromatographic analysis for residual perchloroethylene performed on capsules at different stages in the process: directly off the machine; at the end of the dryer prior to the oven; and after 16 and 32 hours of oven drying. Based on headspace analysis, the capsules contain no detectable level of perchloroethylene residue after drying (detection limit = 0.4-0.5 ppm).

* bacterial count on gelatin capsule after curing - After case hardening, the capsules are subjected to microbiological analyses. The encapsulator performs a pathogen screen and standard plate count. All lots have been accepted with a negative pathogenic screening and a standard plate count of <10 CFU/gm. In addition, the NMFS Charleston Laboratory analyzes the gelatin of the encapsulated oils for coliforms, and *Salmonella* using the procedures presented in the QA Methods Manual (Appendix 2).

FIGURE 6-1. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Hide Gelatin Special Blend

RM Listing Designation: Hide Gelatin (P.C.) BlendRM Code:8626

Specifications:

Description	: Sheets, flakes, shreds or a coarse to fine powder; faintly yellow or amber in color; the color varying in depth according to particular size; slight characteristic bouillonlike odor in solution
Identification	: Positive by visual inspection and compare to type
Assay/Gel Strength	: 150.0 to 165.0 Bloom
Sieve Analysis	: Not less than 100% through a 16 U.S. Standard Sieve Not less than 1% through a 100 U.S. Standard Sieve
LOD	: Not more than 13%
Arsenic	: Not more than 0.8ppm
Heavy Metals	: Not more than 50 ppm (0.005%) (as Pb)
pH	: 5.8 - 6.2
Residue on Ignition	: Not more than 2.0%
Solubility	: Insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, acetic acid, and hot mixtures of glycerin and water; insoluble in alcohol, chloroform, ether, and fixed and volatile oils.
Viscosity	: 41 $\pm$ 3 mps at 60 degrees C
Dioxide	: Not more than 0.15%
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Pathogens : Negative : Standard Plate Count: Less than 10,000 CFU/gm : Mold & Yeast : Less than 5000 CFU/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot : received. The C of A may be used in placed of any of all : testing required above to verify the material meets : specifications.
References	: Vendor Protocol

FIGURE 6-2. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Glycerin - Glycerol 92.09

RM Listing Designation: Glycerin, Natural 99.5%RM Code:8194

Specifications:

Description	: Clear, colorless, syrupy liquid, have a sweet taste. Has not more than s slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic.
Identification	: Positive by IR comparison
*Assay	: NLT 95.0% - NMT 101.0%
*Specific Gravity	: NLT 1.249
Solubility	: Miscible with water, alcohol, and methanol
Insoluble	: Chloroform, ether, and fixed & volatile oils
Color	: Clear, colorless liquid / GNC Gardner Scale 1
*Arsenic	: 1.5 ppm
*Heavy Metals	: Limit is 5 ppm
Loss on Ignition	:
Saponification Value:	
*Chloride	: 0.001%
*Sulfate	: NMT 0.002%
*Fatty Acids and Esters:	NMT 1 ml of 0.5 sodium hydroxide is consumed
*Chlorinated compounds:	(0.003% of Cl)
Iodine Value	:
Unsaponifiable Matter (%)	:
Viscosity	: 995 Cps at 25 C, Brookfield apparatus RVT
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Coliforms : Less than 1,000 MPN/gm : Standard Plate Count: Less than 5000 CFU/gm : Mold & Yeast : Less than 500 CFU/gm : Fecal Coliforms : Less than 100 MPN/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot received. The product code number should also be shown on each container and certificate of analysis forwarded to Quality Control.
References	: U.S. Pharmacopeia XXI, p. 464

* Designates official USP XXI specification

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

The containers used for bottling capsules of n-3 ethyl ester concentrate amber high density linear polyethylene bottles as described in Figure 6-3.

FIGURE 6-3. GENERAL SPECIFICATIONS FOR PLASTIC BOTTLES.

REVISED: JUNE 14, 1985

MEETS USP III CONTAINER SPECIFICATIONS PAGES 1238-1240.

Amber Plastic Bottles

a. Resin - high density, linear polyethylene
b. Pigment - Iron Oxide
c. Mold Release Compound - Zinc Stearate 0.15 percent
d. Flame Treated
e. Style M Necks

MINIMUM WALL THICKNESS	
<500 cc	0.025"
500 cc	0.035"
625 cc	0.035"
750 cc	0.038"
950 cc	0.040"
1300 cc	0.047"

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b. **Labels**

An example of the label used on bottles of capsules of n-3 ethyl ester concentrate appears in Figure 6-4 and contains the following information: BIOMEDICAL TEST MATERIALS, Lot XXXXXXXX, 100 Capsules (1000 mg), Keep Refrigerated, "CAUTION: NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE".

FIGURE 6-4. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF n-3 ETHYL ESTER CONCENTRATE.

3. STORAGE STABILITY

Storage stability of n-3 ethyl ester concentrate in soft gelatin capsules at 5°C is currently being studied. The data is summarized in Table 6-4.

TABLE 6-4. STORAGE STABILITY OF SOFTGEL CAPSULES OF n-3 ETHYL ESTER CONCENTRATE.

	MONTHS STORED				
ANALYSIS TYPE	**0**	**3**	**6**	**9**	**12**
ESTER, %	92	91	90	-	91
EPA, mg/g	414	408	402	-	405
DHA, mg/g	236	229	227	-	229
TOTAL n-3, mg/g	788	758	748	-	750
a-TOCOPHEROL, mg/g	1.5	0.95	0.80	-	1.0
g-TOCOPHEROL, mg/g	2.1	1.67	1.62	-	1.0
PEROXIDE VALUE, meq/kg	1.9	1.82	1.79	2.00	1.97
ANISIDINE VALUE	39.6	37.41	38.5	-	37.8
SENSORY ANALYSIS:					
ODOR:					
TIO	4.96		3.68	5.4	4.53
BUTTERY	0	-	0	0	0
BEANY	0	-	0.13	0	0
RANCID	0	-	0	0	0
PAINTY	1.06	-	0	1.18	1.17
OXIDIZED	0	-	0	0.58	1.52
GRASSY	0.41	-	0	0	0.13
FISHY	0	-	0	0.5	0
BITTER	0.89	-	0.68	1.0	0
SWEET	0	-	0	0	0.2
FRUITY/MELON	1.46	-	1.75	1.53	1.27
BURNT	0	-	0	0	0
SOAPY/SOLVENT	3.17	-	1.93	2.58	0.92
FLAVOR:					
TIF	5.31	-	4.15	5.83	5.95
BUTTERY	0	-	0	0	0
BEANY	0	-	0	0	0
RANCID	0	-	0.45	0	0
PAINTY	1.37	-	0	1.18	1.27
OXIDIZED	0.21	-	0	0.28	2.35
GRASSY	0.37	-	0	0	0.18
FISHY	0.28	-	0.23	1.5	0.3
BITTER	2.04	-	1.35	1.0	1.13
SWEET	0	-	0	0	0
FRUITY/MELON	1.26	-	1.53	0.43	1.42
BURNT	0	-	0	0	0
SOAPY/SOLVENT	5.36	-	1.48	3.03	1.25

C. BULK PACKAGING OF n-3 ETHYL ESTER CONCENTRATE

Researchers conducting animal experimentation primarily utilize n-3 ethyl ester concentrate in bulk form. The concentrate is custom packaged consistent with the research protocol.

1. PROCESS INFORMATION

a. Introduction

One of the simplest techniques for the storage and dispensing of liquids is the use of screw-cap bottles. Screw-cap bottles are satisfactory for dispensing single samples and product stability will be maintained if the material is bottled under nitrogen and stored at low temperatures. However, because bottles are not perfectly amenable to uses requiring multiple-dose dispensing, they require nitrogen purging between uses to maintain an inert atmosphere. The BTM is custom packaged in a manner consistent with its use in the research protocol. The objective of custom packaging is directed at providing the researcher with the quantity of material that will facilitate opening the container a minimum number of times. For instance, the BTM is aliquotted into quantities that facilitate the mode of administration, such as the quantity utilized in the formulation of diet at one time, or the amount of material to be used in gavage studies for a one week period. If there is concern over the use of bulk ethyl ester concentrate in a particular protocol, the Charleston laboratory will conduct a simulated trial of the research protocol to obtain data on the product stability and chemical characteristics when exposed to such a protocol.

b. Process Description

The bulk n-3 ethyl ester concentrate is held in stainless steel tanks under a vacuum or in 55 gal food-grade steel drums under a nitrogen atmosphere. The n-3 ethyl ester concentrate is stored at 5°C for a 3 month period in which the product is used for research requests. At the end of the 3 month period the remaining n-3 ethyl ester is either disposed of or returned to production for reprocessing. The materials are transferred via nitrogen pressure to the specified size and quantity of containers for each researcher. A Wheaton Perifill liquid dispenser is available for delivering small volumes of fish oils and n-3 ethyl ester concentrate. Larger size containers are filled directly from the stainless steel containers. The containers are flushed with nitrogen prior to, and during, the filling process. The bulk oil is well-blanketed with nitrogen prior to capping. The bottles are capped tightly, fastened with tape, placed in plastic bags and tied, in case of spillage during shipping.

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

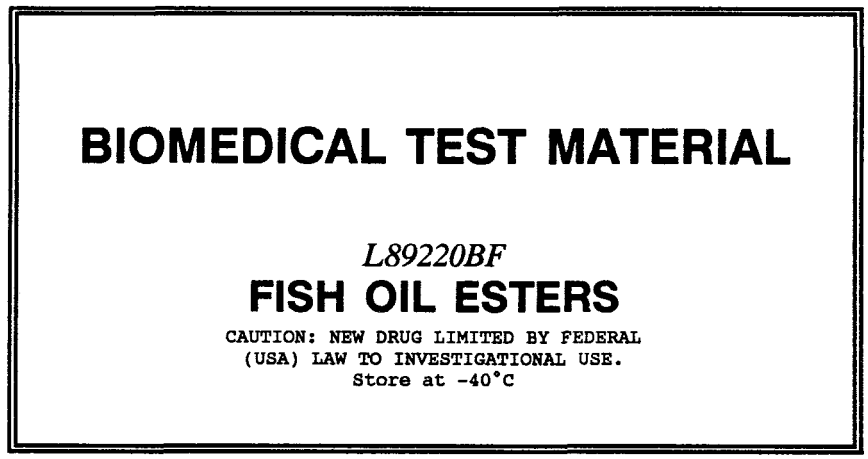
All of the plastic containers presently used for shipment of ethyl esters are FDA approved for food use. The high density polyethylene containers, used for shipment of ester concentrate, are those supplied by Nalgene Corporation, Rochester, NY. The following stock numbers reflect these Nalgene amber, polyethylene food-grade containers:

2004-8125 (4 ml)
2004-9125 (4 ml)
2004-9025 (8 ml)
2004-9050 (15 ml)
2004-0001 (30 ml)
2004-0002 (60 ml)
2009-0004 (125 ml)
2009-0008 (250 ml)
2009-0016 (500 ml)
2009-0032 (1000 ml)
2009-0064 (2000 ml)

b. Labels

Labels currently are being generated using computer fonts and a laser printer, photocopied onto self-adhesive labels and applied to the bulk containers. The label contains the following information: BIOMEDICAL TEST MATERIAL, Lot XXXXXXXX, Store at -40°C, "CAUTION: NEW DRUG LIMITED BY FEDERAL USE (USA) LAW TO INVESTIGATION USE". An example label used on the bulk containers of n-3 ethyl ester concentrates is shown in Figure 6-5.

FIGURE 6-5. EXAMPLE LABEL FOR BULK CONTAINERS OF n-3 ETHYL ESTER CONCENTRATE.



3. STORAGE STABILITY

A storage stability study has been initiated in which n-3 concentrates, containing alpha-tocopherol and TBHQ antioxidants, are being stored at -40°C in 100 ml aliquots in Nalgene (thick-walled polyethylene) bottles. Sampling will be performed quarterly over a period of two years to determine the stability of the ester concentrate, in terms of fatty acid composition, iodine value, free fatty acid content, peroxide value, anisidine value, antioxidant content, and sensory attributes. A summary of the data is presented in Table 6-5.

TABLE 6-5. STORAGE STABILITY OF BULK PACKED n-3 ETHYL ESTER CONCENTRATE STORED AT -40°C.

	MONTHS STORED				
ANALYSIS TYPE	0	3	6	9	12
EPA, mg/g	439	446	429	423	426
DHA, mg/g	240	243	232	226	228
TOTAL n-3, mg/g	783	789	765	761	766
a-TOCOPHEROL, mg/g	0.9	0.9	0.9	0.9	0.9
g-TOCOPHEROL, mg/g	1.3	1.4	1.3	1.3	1.4
PEROXIDE VALUE, meq/kg	5.8	8.5	12.3	9.1	8.1
ANISIDINE VALUE	50.1	48.9	52.11	50.4	51.0
FREE FATTY ACIDS	0.23	_	-	-	-
SENSORY ANALYSIS:					
ODOR:					
TIO	4.28	3.44	4.01	4.3	3.78
BUTTERY	0	0	0	0	0
BEANY	0	0	0	0	0
RANCID	0	0	0	0	0
PAINTY	0.91	1.47	0.9	0.49	0.7
OXIDIZED	0	0	0.13	0	0
GRASSY	0.12	0.18	0.41	0.23	0.15
FISHY	0	0	0.06	0	0
BITTER	0.72	0.43	0.67	0.19	0.55
SWEET	0	0	0	0	0
FRUITY/MELON	1.37	1.09	0.69	1.83	1.13
BURNT	0	0	0	0	0
SOAPY	1.32	0.83	0.98	1.41	1.78
SOLVENT	0.9	1.08	1.42	0.83	1.18
FLAVOR:					
TIF	4.33	4.42	4.15	4.47	3.93
BUTTERY	0	0	0	0	0
BEANY	0.12	0.14	0	0.21	0
RANCID	0	0	0	0	0
PAINTY	0.4	1.12	0.97	0.68	0.78
OXIDIZED	0	0.23	0.1	0.12	0
GRASSY	0.31	0.06	0.99	0.56	0
FISHY	0.35	0.08	0.79	0.36	0
BITTER	1.31	2.02	0.96	0.78	2.03
SWEET	0	0	0	0	0
FRUITY/MELON	0.88	0.7	0.24	1.17	1.48
BURNT	0	0	0	0	0
SOAPY	1.02	3.36	1.56	2.01	2.28
SOLVENT	1.36	0.82	1.2	1.84	2.55

D. BULK PACKAGING OF ETHYL ESTERS of PURIFIED EPA and DHA

These materials will be supplied to individuals conducting basic research into the mechanism of action of specific polyunsaturated fatty acids. The studies will utilize *in vitro* systems primarily. Bulk packaging in milligram to gram quantities will be required by these studies.

1. PROCESS INFORMATION

a. Special Handling Conditions

Purified esters are transferred from the receiver of the product separator to N_2-purged brown glass bottles with Teflon caps, and stored at -40°C. Dispensing of the materials for distribution takes place in a glove box that has been well purged with N_2 and through which N_2 continues to flow during the operation. Materials are transferred from bulk storage containers to brown glass or plastic vials sized for one-time use of the contents, using disposable Pasteur pipettes. Crimp-top or screw-on caps with Teflon liners are used. The materials are packed with dry ice for shipment to investigators.

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

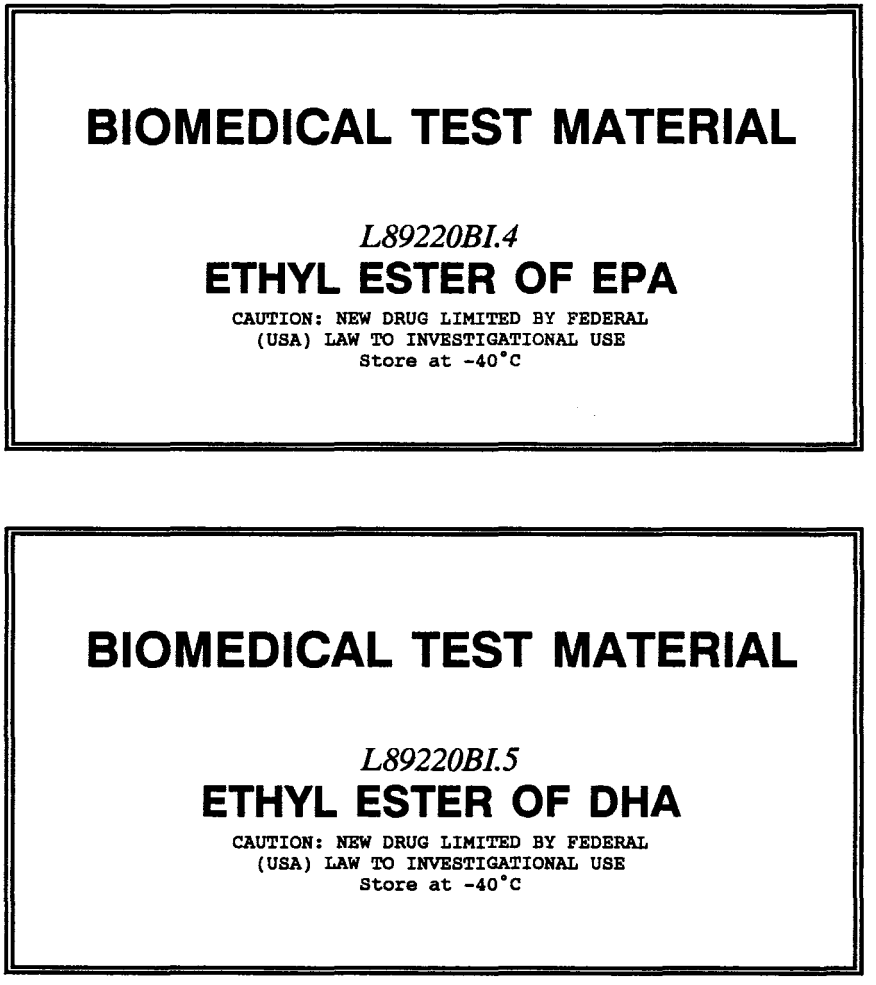
Containers used to store purified EPA and DHA are amber in color and made of high density polyethylene. Since the containers (both vials and bottles) are leakproof they are well suited for storage and shipment of liquid materials. The caps are amber polyethylene screw-type closures. The containers are available in a variety of sizes:

Container	Manufacturer	Stock Number
3.8 ml	Nalge	2004-9125
4 ml	Nalge	2004-8125
8 ml	Nalge	2004-9025
15 ml	Nalge	2004-9050
30 ml	Nalge	2004-0001
60 ml	Nalge	2004-0002
125 ml	Nalge	2009-0004

b. Labels

Labels currently are being generated using computer fonts and a laser printer, photocopied onto self-adhesive labels and applied to the bulk containers. The label contains the following information: BIOMEDICAL TEST MATERIAL, Lot XXXXXXXX, Store at -40°C, "CAUTION: NEW DRUG LIMITED BY FEDERAL USE (USA) LAW TO INVESTIGATION USE". An example label used on bulk containers of n-3 ethyl esters of EPA and DHA is shown in Figure 6-6.

FIGURE 6-6. EXAMPLE LABELS FOR BULK CONTAINERS OF PURIFIED EPA AND DHA.



3. STORAGE STABILITY

The storage stability of purified EPA is currently under study and will be reported at a later date. Two different types of storage containers and two temperatures are being investigated. Sampling will be performed over a year with more frequent samplings in the early phase. Parameters under study are delineated in Table 6-6. The integrity of the samples will be determined in terms of fatty acid data (qualitative and quantitative data obtained from TLC and GLC), peroxide values, tocopherol levels, sensory analysis and moisture content. Baseline data are shown in Table 6-6.

TABLE 6-6. STORAGE STABILITY OF PURIFIED EPA STORED AT -40° C IN EITHER HIGH-DENSITY POLYETHYLENE OR GLASS.

	MONTHS STORED								
	0	1		3		4		6	
ANALYSIS TYPE		GLASS	PE	GLASS	PE	GLASS	PE	GLASS	PE
ESTERS, %	92.8	93.1	92.9	93.5	93.4	93.6	95.2	94.4	95.0
EPA, mg/g	902	904	898	908	908	911	925	916	922
Peroxide Value, meq/kg	1.61	1.68	1.67	1.38	1.82	1.67	1.23	1.28	2.62
a-Tocopherol, mg/g	0.37	-	-	-	-	-	-	-	-
g-Tocopherol, mg/g	1.38	-	-	-	-	-	-	-	-
Moisture, ug/g	1559	-	-	-	-	-	-	-	-
Sensory Analysis:									
TIO	2.74	-	-	-	-	-	-	-	-
TIF	3.00	-	-	-	-	-	-	-	-

* Additional data to be collected at 12 months.

SECTION VII. PROCESSING/CHEMICAL COMPOSITION DATA FOR ETHYL ESTERS OF VEGETABLE OILS: CORN, OLIVE, AND SAFFLOWER.

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C. BULK PACKAGING of ETHYL ESTERS of VEGETABLE OILS

1. PROCESS INFORMATION
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A. GENERAL INFORMATION

1. STARTING MATERIAL SPECIFICATIONS

a. Corn Oil

Corn oil is the refined fixed oil obtained from the embryo of ***Zea mays*** Linne' (Fam. Gramineae). It is a clear, light yellow, oily liquid. It has a faint, characteristic odor and taste. It is slightly soluble in alcohol and miscible with ether, chloroform, benzene and petroleum ether. The quality specifications for this oil are included in Table 7-1.

b. Olive Oil

Olive oil is the fixed oil obtained from the ripe fruit of ***Olea europaea*** Linne' (Fam. Oleaceae). It is pale yellow, or light greenish yellow, oily liquid having a slight characteristic odor and taste, with a faintly acrid after-taste. It is slightly soluble in alcohol and is miscible with ether, chloroform, and carbon disulfide. The quality specifications for this oil are included in Table 7-1.

c. Safflower Oil

Safflower oil is the refined fixed oil obtained from the seed of ***Carthamus tinctorius*** Linne' (Fam. Compositae). It is a clear, light yellow, oily liquid. It has a faint, characteristic odor and taste. It is slightly soluble in alcohol and miscible with ether, chloroform, benzene and petroleum ether. The quality specifications for this oil are included in Table 7-1.

2. PROCESSING INFORMATION

a. Production of Ethyl Esters of Vegetable Oils

The feedstock for production of ethyl esters of vegetable oils is commercially deodorized food grade corn, olive, or safflower oils, described above in 'Starting Material Specifications'. The first step in production of the ethyl esters of vegetable oils is the preparation of fatty acid ethyl esters using absolute undenatured ethanol and sodium ethoxide synthesized in the production plant from metallic sodium and absolute ethanol. Two glass reactors (72 L) are used to prepare 80 Kg of ethyl esters (40 Kg in each reactor) from 80 Kg of deodorized oil in a N_2 atmosphere. After reaction is complete, the solution is allowed to stand until it separates into two phases. The lower alcoholic glycerol phase is drained off and the upper phase, consisting of the esters and some alcohol, is pumped into a 100 L glass separatory funnel, under N_2, where the esters are washed several times with deionized water. Following the final wash, the esters are left overnight in the funnels to allow complete separation of the water from the esters. Prior to transferring the esters to their storage vessel, any visible water in the separatory funnel is drained and the moisture content determined by an automated Karl Fischer method.

Storage containers consist of 36-gal stainless steel pressure vessels. Once the esters are transferred to these containers, the vessels are evacuated using a vacuum pump until the measured moisture in the esters is <500 ug/g.

TABLE 7-1. QUALITY SPECIFICATIONS FOR VEGETABLE OILS TO BE USED AS STARTING MATERIALS FOR TRANSESTERIFICATION.

	VEGETABLE OIL		
ANALYSIS TYPE	**Corn**	**Olive**	**Safflower**
TRIGLYCERIDES, %	>95	>95	>95
16:0, %	8-12	9-17	6-7
18:1n-9, %	19-49	50-84	9-14
18:2n-6, %	34-62	4-18	76-81
FREE FATTY ACIDS, %	<0.2	<0.2	<0.2
CHOLESTEROL, mg/g	0	0	0
PEROXIDE VALUE (meq/kg)	<10.0	<10.0	<10.0
IODINE VALUE, g I2/100g	102-130	79-88	135-150
ANISIDINE VALUE	<20	<20	<20
a-TOCOPHEROL, mg/g	0.1-1.0	0.1-1.0	0.1-1.0
g-TOCOPHEROL, mg/g	0.1-1.0	0.05-0.5	0.05-0.5
MOISTURE, ug/g	<500	<500	<500
PCBs, ug/g	<0.5	<0.5	<0.5
TOTAL DDT, ug/g	<0.5	<0.5	<0.5
TRACE METALS, ug/g:			
Arsenic	<1.0	<1.0	<1.0
Cadmium	<1.0	<1.0	<1.0
Lead	<1.0	<1.0	<1.0
Mercury	<1.0	<1.0	<1.0
Selenium	<1.0	<1.0	<1.0
SENSORY ATTRIBUTES:			
ODOR (TIO)	<4.0	<4.0	<4.0
FLAVOR (TIF)	<4.0	<4.0	<4.0
OTHER:			
SPECIFIC GRAVITY	.914-.921	.910-.915	.919-.924
SOLIDIFICATION RANGE	-10 to -6°C	-8 to -3°C	-18 to -16°C
SAPONIFICATION VALUE	187-193	190-195	186-194
UNSAPONIFIABLE MATTER	<1.5%	<1.5%	<1.5%

* TIO= Total intensity of odor, TIF= Total intensity of flavor.

b. Specifications for Chemicals, Solvents and Additives Used in the Process:

* Tocopherols and antioxidant TBHQ - The tocopherols (alpha- and gamma-tocopherol) and the antioxidant tertiary butylhydroquinone (TBHQ) are added to the ethyl esters of vegetable oil at the Charleston Laboratory so that the final product has approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, 0.02% TBHQ. TBHQ is added as a mixture containing the components listed below. The tocopherols and the TBHQ are added to increase the stability and shelf life of the test materials and to achieve a balance of these components between the placebo ester and the n-3 ester concentrate. The objective is to attain approximately 1.0 mg/g for both alpha- and gamma-tocopherol. The actual values in soft-gel capsules will vary depending upon a number of factors such as experimental error associated with weighing and mixing the chemicals, as well as analytical methodology. Tocopherols contained in the ethyl esters of vegetable oils are those naturally occurring and those added after transesterification to attain a balanced level with the complimentary n-3 ester concentrate test material. The added components consist of Vitamin E 5-67, Tenox GT-1, and Tenox 20A (all products are from Eastman Chemical Co., Rochester, NY). These products have been analyzed at the Charleston Laboratory and will be tested for identity each time a new lot is received, prior to addition to processed oils. The products contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monooleate 32% Propylene glycol 15% Corn oil 30%
Tenox GT-1	Mixed tocopherols (alpha, gamma, delta) 50% Vegetable oil 50%.
Vitamin E 5-67	672 mg/g d-alpha tocopherol in edible soybean oil (guaranteed to contain >95% of the tocopherols as d-alpha).

* Absolute undenatured ethanol - USP grade, anhydrous, 200 proof.

* Metallic sodium - ACS grade packed under vacuum.

* Gaseous nitrogen - FDA approved and < 5 ppm O_2.

* Hydrochloric acid - ACS grade.

* Deionized water - >16.7 megaohm-cm resistance.

* Alkali (KOH) - technical grade.

3. EQUIPMENT MAINTENANCE

Specific cleaning methods, including frequency, are described in the Production Methods and Safety Manual (Appendix 1). All major production equipment is cleaned with materials described above (undenatured alcohol, deionized water, and alcoholic alkali). All glassware and stainless-steel product receivers are cleaned with detergent (described below) followed by thorough rinsing with tapwater, deionized water, and undenatured absolute ethanol.

* The specification for the detergent used in cleaning the production equipment including the product receivers is biodegradable, USDA/FDA approved for use in the food industry. Brand names are "Simple Green" and "Planisol-M".

4. CHEMICAL COMPOSITION AND QUALITY ASSURANCE

Quality specifications for the ethyl esters of vegetable oils produced at Charleston Laboratory are given in Table 7-2. Since these values are not provided in the standard fats and oils literature, they represent the specifications set by the Charleston Laboratory Quality Assurance Project based on practical experience with fish oil ethyl esters.

The ethyl esters of vegetable oils have been extensively analyzed by the Quality Assurance/Quality Control Project at the Charleston Laboratory for several categories of analysis: lipid classification, sterols, fatty acid oxidation products, organics, metals, moisture, and sensory attributes (Table 7-3a). The complete fatty acid composition of the ethyl esters have been determined by capillary gas chromatography (Table 7-3b). The methods used for all analyses are described in the NOAA Technical Memorandum NMFS-SEFC-211, "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

Peroxide value, iodine value, and moisture content were determined using automated titrators. Anisidine value, which is analyzed by standard IUPAC methods, is a measure of aldehydes. Tocopherols were analyzed to assure the concentrations added and to provide a measure of vitamin E activity.

The esters were analyzed for six potentially toxic metals: As, Se, Hg, Cd, Pb, and Ni. The products were also analyzed for eight macroelements which are either of nutritional interest or may potentially be picked up during processing.

The fatty acid composition was determined by comparison of specific fatty acid relative retention time (RRT) with primary and secondary standards, by hydrogenation, argentation TLC, and GC/MS of their methyl esters. The method is described in detail in Appendix 2.

The products were subjected to sensory analysis by a trained sensory evaluation panel for total intensity of odor (TIO), total intensity (TIF) of flavor, and eleven different odor/flavor attributes. The values reported are based on an unstructured scale 15-cm in length, on which "0" represents "absent" and "15" represents "very strong" presence of a given attribute. The scores obtained on these products represent a rather mild odor/flavor profile.

TABLE 7-2. QUALITY SPECIFICATIONS FOR ETHYL ESTERS OF VEGETABLE OILS (PLACEBOS) PRODUCED AT THE CHARLESTON LABORATORY.

	ETHYL ESTER		
ANALYSIS TYPE	**CORN**	**OLIVE**	**SAFFLOWER**
ESTERS, %	>85	>85	>85
16:0, %	8-12	9-17	6-7
18:1n-9, %	19-49	50-84	9-14
18:2n-6, %	34-62	4-18	76-81
FREE FATTY ACIDS, %	<0.2	<0.2	<0.2
CHOLESTEROL, mg/g	<0.05	<0.05	<0.05
PEROXIDE VALUE, meq/kg	<10.0	<10.0	<10.0
IODINE VALUE, g I2/100g	80-120	60-100	100-150
ANISIDINE VALUE	<20	<20	<20
a-TOCOPHEROL, mg/g	0.5-5.0	0.5-5.0	0.5-5.0
g-TOCOPHEROL, mg/g	0.5-5.0	0.5-5.0	0.5-5.0
TBHQ, mg/g	0.1-0.2	0.1-0.2	0.1-0.2
MOISTURE, mg/g	<2	<2	<2
PCBs, ug/g	<0.5	<0.5	<0.5
TOTAL DDT, ug/g	<0.5	<0.5	<0.5
TRACE METALS, ug/g:			
Arsenic	<1.0	<1.0	<1.0
Cadmium	<1.0	<1.0	<1.0
Lead	<1.0	<1.0	<1.0
Mercury	<1.0	<1.0	<1.0
Selenium	<1.0	<1.0	<1.0
SENSORY ATTRIBUTES:			
ODOR (TIO)*	<7.0	<7.0	<7.0
FLAVOR (TIF)*	<7.0	<7.0	<7.0
OTHER:			
SPECIFIC GRAVITY	0.86	0.86	0.86
SOLIDIFICATION RANGE	**	**	**

* TIO= Total intensity of odor, TIF= Total intensity of flavor.

** Liquid at 5°C or higher.

TABLE 7-3a. CHEMICAL COMPOSITION OF ETHYL ESTERS OF VEGETABLE OILS (PLACEBOS).

ANALYSIS TYPE	ETHYL ESTER CORN L89165VF	OLIVE L88333WF	SAFFLOWER L89194YF
TRIGLYCERIDE, %	*	*	*
ETHYL ESTERS, %	88	88	91
16:0, %	10	10	6
18:1n-9, %	23	67	8
18:2n-6, %	50	6	72
TOTAL n-3, mg/g	5.9	6	1.8
FREE FATTY ACIDS, %	0.07	0.09	**
TRANS FATTY ACIDS, %	<5	**	**
CHOLESTEROL, mg/g	<0.05	<0.05	<0.05
PEROXIDE VALUE, meq/kg	3.17	2.3	3.43
IODINE VALUE, g I2/100g	110.7	86.6	141
ANISIDINE VALUE	5.7	4.78	8.0
ANTIOXIDANT CONTENT:			
a-TOCOPHEROL, mg/g	1.5	1.1	1.5
g-TOCOPHEROL, mg/g	2.1	1.0	2.3
MOISTURE, ug/g	502	422	1692
PCBs, ug/g	**	**	**
TOTAL DDT, ug/g	**	**	**
SENSORY ATTRIBUTES,			
0-15, 15 MAX INTENSITY:			
ODOR:			
TIF	3.45	5.09	4.28
BUTTERY	0	0	0
BEANY	0	0.21	0
RANCID	0	0.06	0
PAINTY	0.13	0.94	0.35
OXIDIZED	0	0.1	0
GRASSY	0	0.47	0
FISHY	0	0	0
BITTER	0.13	0.2	0.63
SWEET	0.4	0.3	0.68
FRUITY/MELON	0	0.4	1.6
BURNT	0	0	0
ALMOND/RUM/ALCOHOL	1.63	0	1.25
CARDBOARD	0.75	0	0.8
SOAPY	0	1.63	0.23
SOLVENT	0	1.54	0.8

TABLE 7-3a. CONTINUED.

ANALYSIS TYPE	ETHYL ESTER CORN L89165VF	ETHYL ESTER OLIVE L88333WF	ETHYL ESTER SAFFLOWER L89194YF
FLAVOR:			
TIF	3.85	5.04	4.4
BUTTERY	0	0	0
BEANY	0	0.04	0
RANCID	0.23	0.81	0.5
PAINTY	0	1.1	0
OXIDIZED	0	0	0.43
GRASSY	0	0.63	0
FISHY	0	0.44	0
BITTER	0.3	0.29	0.9
SWEET	0.4	0.48	0.68
FRUITY/MELON	0	0.13	1.88
BURNT	0	0.29	0
ALMOND/RUM/ALCOHOL	1.7	0	0.98
CARDBOARD	0.43	0	0.5
SOAPY	0	2.92	0.48
SOLVENT	0	2.44	0.6
OTHER:			
SPECIFIC GRAVITY	0.86	0.86	0.86
SOLIDIFICATION RANGE	***	***	***

* The non-ethyl ester portion consists of triglycerides, mono- and diglycerides, unsaponifiables, sterols and wax esters.

** Not analyzed.

*** Liquid at 5°C or higher.

TABLE 7-3b. FATTY ACID COMPOSITION (mg/g) OF ETHYL ESTERS OF VEGETABLE OILS.

	ETHYL ESTER*		
FATTY ACID	OLIVE L89165WF	SAFFLOWER L88333YF	CORN L89194VF
14:0	0**	0.9	0.3
16:0	101.8	57.6	102.9
17:0	0.8	0.1	0.7
18:0	23.5	18.2	19.0
20:0	4.3	2.9	4.1
22:0	2.0	2.5	1.4
24:0	4.7	1.4	0.8
TOTAL SATS.	137.1	83.6	129.2
16:1n9	1.1	0.0	0.4
16:1n7	8.6	0.8	1.1
17:1	1.6	0.0	0.3
18:1n9	604.9	84.2	228.9
18:1n7	21.6	6.3	5.8
20:1n9	3.7	1.5	2.3
TOTAL MONOENES	641.5	92.9	238.8
18:2n6	93.7	720.2	501.1
TOTAL DIENES	93.7	720.2	501.1
18:3n3	6.0	1.8	5.9
TOTAL PUFA	99.7	721.9	507.0
TOTAL n-3	6.0	1.8	5.9
TOTAL n-6	93.7	720.2	501.1
n-3/n-6	0.1	0.0	0.0

* Ethyl esters were identified by comparison of their RRTs with those of primary and secondary standards, by hydrogenation, argentation TLC, and GC/MS of their ethyl esters.

** 0.0 = <0.05 mg/g

5. TECHNICAL SUPPORT

Technical support for the researchers by BTMP personnel includes a discussion of the experimental protocol. Input from the BTMP is focused on the development of the best protocol for the utilization of the test materials while maintaining their high quality. Each researcher approved for the requested amount of Biomedical Test Material (BTM) by the Fish Oil Test Material Distribution Committee (FOTMDC) receives a telephone call soon after receipt of the approval letter. The initial conversation covers a diversity of topics depending upon the research need and protocol. The information in the application is verified in terms of research objective, experimental protocol, and the mode of administration. Information on the availability, composition, storage, and stability data of the BTM is provided. The amount of technical assistance provided to each researcher varies, depending upon their specific needs. Some researchers have significant experience in the n-3 research field and require less assistance than those just entering the n-3 arena. Technical support is provided in the forms listed below:

a. Suitability of type and quantity of requested BTM for proposed research protocol.

b. Literature information obtained from the Fish Oil Bibliography.

c. Analytical data, including the Quality Assurance data.

d. Antioxidants present in the BTM and balancing of placebo test materials including analysis of placebo oils.

e. Use of control/placebo treatments in experimental designs; flavoring/masking of placebo treatments in double blind trials.

f. Customized packaging and scheduling of shipments of BTM.

Additional information is provided the researcher in the form of printed materials and includes:

g. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil" (Appendix 2).

h. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules" (Appendix 4).

i. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil" (Appendix 5).

j. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids.

k. Technical information on the proper storage of each of the test materials to prevent their oxidative deterioration and to ensure maintenance of the high quality of the delivered test material.

l. Technical information is provided in support of IND (Investigative New Drug) applications: The use of BTM in human studies requires the attainment of an IND number from the Food and Drug Administration. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. This

Drug Master File (DMF) containing technical information on the chemical composition, processing, and handling of the specific test materials serves to expedite IND requests for researchers approved for using the BTM in their studies. The NIH/Fish Oil Test Material Program (FOTMP) office submits the names of approved researchers planning human studies to the FDA to authorize access to the appropriate DMF.

m. NOAA Technical Memorandum NMFS-SEFC-234; "Biomedical Test Materials Program: Production Methods and Safety Manual" (Appendix 1).

B. ENCAPSULATION of ETHYL ESTERS of VEGETABLE OILS

Researchers involved in human studies usually use fish oil in the soft-gelatin capsule form. On rare occasion, capsules are utilized to introduce test materials to primates. They are also ideal 'containers' for storage of secondary analytical standards.

1. SOFT GELATIN ENCAPSULATION PROCESS INFORMATION

a. Introduction

The rotary die process, invented by R.P. Scherer in 1933, is a continuous process for producing soft gelatin capsules. The rotary die process reduced manufacturing losses to a negligible figure and content variation to $\pm$ 1-3%. Capsules are manufactured and partially dried in the following three continuous steps:

* Two gelatin ribbons are prepared, automatically and continuously, and fed, with the fill material, to the encapsulating mechanism.
* The capsules are simultaneously and continuously filled, with the force of the injected fill material causing the gelatin to expand into the die pockets to form the shape of the product, hermetically sealed and automatically cut between two rotary dies.
* The formed capsules are automatically conveyed to and through a solvent wash unit and partially dried in a forced-air tunnel.

The gelatin contains approximately 30 percent water and is heated to a temperature of 37-40°C. The physical characteristics of gelatin are related to the (1) "bloom", a measure of the cohesive strength of the cross-linking, which occurs between gelatin molecules and is proportional to the molecular weight of the gelatin, and (2) viscosity, a measure of the molecular chain length which determines the manufacturing characteristics of the gelatin film.

After the capsules are formed and washed with solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5% of the water. The product is then subjected to drying in a forced air oven (24% R.H., 70°F) for 16 hours which produces a capsule with 6-10% water content at equilibrium.

b. Process Description

Ethyl esters of vegetable oils are encapsulated at General Nutrition Corporation (GNC), Greenville, SC with a fill weight of 1,000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. The uniformity of dosage units utilized conforms with USP XXI specifications (pp. 1277-1278). A NMFS BTM program representative inspects and provides oversight at encapsulation facilities during encapsulation. It appears that good manufacturing practices are being utilized at the facility during encapsulation of the test materials. Several modifications of the encapsulation process are performed to minimize the contact of the BTM with oxygen and maintain their high quality. The following precautions are taken during encapsulation of the test materials.

* Initial transfer of the fill material from the shipping drum into the process container, routinely consists of the drum being lifted and the contents emptied, causing significant mixing of air into the product. This procedure is altered so that the BTM's are transferred via a transfer line using nitrogen pressure to the process container flushed with nitrogen. The container is blanketed with nitrogen and closed with an air-tight cover during mixing prior to encapsulation.
* During the packaging of the test materials, nitrogen flushing of the bottled capsules is incorporated at the bottle slow down point (3 sec flush time), prior to capping.
* Brown, polyethylene bottles are used to provide the best protection for the test materials while reducing shipping weight of the product.

These test materials are maintained in a nitrogen atmosphere in temperature controlled containers prior to encapsulation. After encapsulation, the bottled capsules are flushed with nitrogen prior to application of the inner heat seal. Labels are applied on line and are described in Section VII-B.2.b of this document. The capsules are stored at 5°C at the Charleston Laboratory.

c. Chemical Composition of Encapsulating Materials

The components used by GNC in formulating the capsule material are gelatin, water, and glycerin. In addition the capsules are rinsed with perchloroethylene. Information regarding the composition of these components is presented below.

* gelatin - Information is provided in Figure 7-1.
* glycerin - Information is provided in Figure 7-2.
* perchloroethylene - meets USP specification requirements for internal use in humans, as stated by the encapsulator.

d. Quality Assurance of Encapsulating Materials

* gelatin - Information is provided in Figure 7-1.

* glycerin - Information is provided in Figure 7-2.

* perchloroethylene residue - The test method consisted of perchloroethylene residue headspace analysis performed on capsules at different stages in the process: directly off the machine; at the end of the dryer prior to the oven; and after 16 and 32 hours of oven drying. Based on a headspace analysis method, the capsules contain no detectable level of perchloroethylene residue after drying (equal to or below the detection limit of "background noise" which is approximately 0.4-0.5 ppm).

* bacterial count on gelatin capsule after curing - After case hardening, the capsules are subjected to microbiological analyses. The encapsulator performs a pathogen screen and standard plate count. All lots have been accepted with a negative pathogenic screening and a standard plate count of <10 CFU/gm. In addition, the NMFS Charleston Laboratory analyzes the gelatin of the encapsulated oils for coliforms and *Salmonella* using the procedures presented in the QA Methods Manual (Appendix 2).

FIGURE 7-1. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Hide Gelatin Special Blend

RM Listing Designation: Hide Gelatin (P.C.) Blend **RM Code:8626**

Specifications:

Description	: Sheets, flakes, shreds or a coarse to fine powder; faintly yellow or amber in color; the color varying in depth according to particular size; slight characteristic bouillon-like odor in solution
Identification	: Positive by visual inspection and compare to type
Assay/Gel Strength	: 150.0 to 165.0 Bloom
Sieve Analysis	: Not less than 100% through a 16 U.S. Standard Sieve Not less than 1% through a 100 U.S. Standard Sieve
LOD	: Not more than 13%
Arsenic	: Not more than 0.8ppm
Heavy Metals	: Not more than 50 ppm (0.005%) (as Pb)
pH	: 5.8 - 6.2
Residue on Ignition	: Not more than 2.0%
Solubility	: Insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, acetic acid, and hot mixtures of glycerin and water; insoluble in alcohol, chloroform, ether, and fixed and volatile oils.
Viscosity	: 41 $\pm$ 3 mps at 60 degrees C
Dioxide	: Not more than 0.15%
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Pathogens : Negative : Standard Plate Count: Less than 10,000 CFU/gm : Mold & Yeast : Less than 5000 CFU/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot : received. The C of A may be used in place of any of all : testing required above to verify the material meets : specifications.
References	: Vendor Protocol

FIGURE 7-2. GENERAL NUTRITION SPECIFICATION SHEET.

Raw Material Nomenclature: Glycerin - Glycerol 92.09

RM Listing Designation: Glycerin, Natural 99.5% **RM Code:8194**

Specifications:

Description	: Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic.
Identification	: Positive by IR comparison
*Assay	: NLT 95.0% - NMT 101.0%
*Specific Gravity	: NLT 1.249
Solubility	: Miscible with water, alcohol, and methanol
Insoluble	: Chloroform, ether, and fixed & volatile oils
Color	: Clear, colorless liquid / GNC Gardner Scale 1
*Arsenic	: 1.5 ppm
*Heavy Metals	: Limit is 5 ppm
Loss on Ignition	:
Saponification Value:	
*Chloride	: 0.001%
*Sulfate	: NMT 0.002%
*Fatty Acids and Esters:	NMT 1 ml of 0.5 sodium hydroxide is consumed
*Chlorinated compounds:	(0.003% of Cl)
Iodine Value	:
Unsaponifiable Matter (%)	:
Viscosity	: 995 Cps at 25 C, Brookfield apparatus RVT
Microbial Limits	: Salmonella : Negative E. Coli: Negative : Coliforms : Less than 1,000 MPN/gm : Standard Plate Count: Less than 5000 CFU/gm : Mold & Yeast : Less than 500 CFU/gm : Fecal Coliforms : Less than 100 MPN/gm
Stability	: Minimum 12 months at Room Temperature (Bulk Storage)
Storage	: Store in tight containers in a cool, dry area
Comments	: A vendor Certificate of Analysis is required with each lot received. The product code number should also be shown on each container and certificate of analysis forwarded to Quality Control.
References	: U.S. Pharmacopeia XXI, p. 464

* Designates official USP XXI specification

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

The containers used for bottling capsules of ethyl esters of vegetable oils are high density amber linear polyethylene bottles as described in Figure 7-3. These specifications were provided by the encapsulator, GNC.

FIGURE 7-3. GENERAL SPECIFICATIONS FOR PLASTIC BOTTLES.

REVISED: JUNE 14, 1985

MEETS USP III CONTAINER SPECIFICATIONS PAGES 1238-1240.

Amber Plastic Bottles

a. Resin - high density, linear polyethylene
b. Pigment - Iron Oxide
c. Mold Release Compound - Zinc Stearate 0.15 percent
d. Flame Treated
e. Style M Necks

MINIMUM WALL THICKNESS

<500 cc	0.025"
500 cc	0.035"
625 cc	0.035"
750 cc	0.038"
950 cc	0.040"
1300 cc	0.047"

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b. Labels

An example label used on bottles of capsules of ethyl esters of vegetable oils appears in Figure 7-4 and contains the following information: BIOMEDICAL TEST MATERIALS, LOT XXXXXXXX, 100 Capsules (1000 mg), Keep Refrigerated, "Caution: New Drug Limited by Federal (USA) Law to Investigational Use".

FIGURE 7-4. EXAMPLE LABEL FOR BOTTLES OF SOFTGEL CAPSULES OF ETHYL ESTERS OF VEGETABLE OILS.

3. STORAGE STABILITY of SOFTGEL ENCAPSULATED ETHYL ESTERS of VEGETABLE OILS

Stability of ethyl esters of olive oil in soft gelatin capsules stored at 5°C is currently being studied. The data are summarized in Table 7-4.

TABLE 7-4. STORAGE STABILITY OF SOFTGEL CAPSULES OF ETHYL ESTERS OF OLIVE OIL.

	MONTHS STORAGE				
ANALYSIS TYPE	0	6	12	18*	24
EPA, mg/g	<0.01	<0.01	<0.01		
DHA, mg/g	<0.01	<0.01	<0.01		
TOTAL n-3, mg/g	6	6.1	6.2		
16:0, mg/g	101.8	104.5	102.5		
18:1n9, mg/g	604.9	610.4	624.8		
18:2n6, mg/g	93.7	94.6	96.9		
a-TOCOPHEROL, mg/g	1.1	0.9	0.9		
g-TOCOPHEROL, mg/g	1.0	0.9	0.9		
PEROXIDE VALUE, meq/kg	2.30	1.79	3.30		
ANISIDINE VALUE	4.78	5.71	5.87		
SENSORY ANALYSIS:					
ODOR:					
TIO	5.09	4.03	4.88		
BUTTERY	0	0	0		
BEANY	0.21	0	0.08		
RANCID	0.06	0	0.17		
PAINTY	0.94	0.13	0.17		
OXIDIZED	0.1	0	0		
GRASSY	0.47	0.13	0		
FISHY	0	0	0		
BITTER	0.2	0	0		
SWEET	0.3	0	0.52		
FRUITY/PERFUMY	0.4	0	0.93		
BURNT	0	0	0		
ALMOND/RUM/ALCOHOL	1.67	1.88	1.43		
SOAPY/SOLVENT	0.32	1.53	0.17		
CARDBOARD	0.42	1.2	2.83		
FLAVOR:					
TIF	5.04	4.45	5.37		
BUTTERY	0	0	0		
BEANY	0.04	0	0.03		
RANCID	0.81	0.28	0		
PAINTY	1.1	0.5	0.22		
OXIDIZED	0	0	0.33		
GRASSY	0.63	0	0.28		
FISHY	0.44	0	0		
BITTER	0.29	1.35	0		
SWEET	0.48	0	0		
FRUITY/PERFUMY	0.13	0	0.72		
BURNT	0.29	0	0		
ALMOND/RUM/ALCOHOL	1.22	1.88	0.98		
SOAPY/SOLVENT	1.17	0.78	0.23		
CARDBOARD	0.44	1.65	3.05		

* Data to be collected 5/90.

C. BULK PACKAGING of ETHYL ESTERS of VEGETABLE OILS

Researchers conducting animal experimentation primarily utilize ethyl ester test materials in the bulk form. Therefore placebo ethyl esters of vegetable oils are provided in bulk also. The ethyl esters of vegetable oils are custom packaged consistent with the research protocol and the packaging of the n-3 ethyl ester concentrate.

1. PROCESS INFORMATION

a. Introduction

One of the simplest techniques for the storage and dispensing of liquids is the use of screw-cap bottles. Screw-cap bottles are satisfactory for dispensing single samples, and product stability will be maintained if the material is bottled under nitrogen and stored at low temperatures. However, because bottles are not perfectly amenable to uses requiring controlled-dose dispensing, they require nitrogen blanketing between uses to maintain an inert atmosphere. Assessment of the research protocol assists in the determination of the best packaging regime to minimize the number of times each container is opened. The BTM is custom packaged in a manner consistent with its use in the research protocol. The objective of custom packaging is directed at providing the researcher with the quantity of material that will facilitate opening the container a minimum number of times. If there is concern over the use of bulk ethyl esters of vegetable oils in a particular protocol, the Charleston Laboratory will conduct a simulated trial of the research protocol to obtain data on the product stability and chemical characteristics when exposed to such a protocol.

b. Process Description

The bulk ethyl esters of vegetable oils are held in 30 gal stainless steel pressure vessels under a vacuum or in 55 gal food-grade steel drums under a nitrogen atmosphere. The ethyl ester test material is stored for a 3-month period at -40° C in which the product is used for research requests. At the end of the 3-month period the remaining ethyl ester is either disposed of or returned to production for reprocessing. This period of storage may be lengthened once sufficient storage study information is collected to warrant this option. The decision will depend upon the peroxide value exceeding the quality specification given in Table 7-2. The materials are transferred via nitrogen pressure to the specified size and quantity of containers for each researcher. A Wheaton Perifill liquid dispenser is available for delivering small volumes of test materials. Larger size containers are filled directly from the product storage vessels. The containers are flushed with nitrogen prior to, and during, the filling process. The bulk esters are well-blanketed with nitrogen prior to capping. The bottles are capped tightly, fastened with tape, placed in plastic bags and tied, in case of spillage during shipping.

2. PACKAGING MATERIALS SPECIFICATIONS

a. Containers

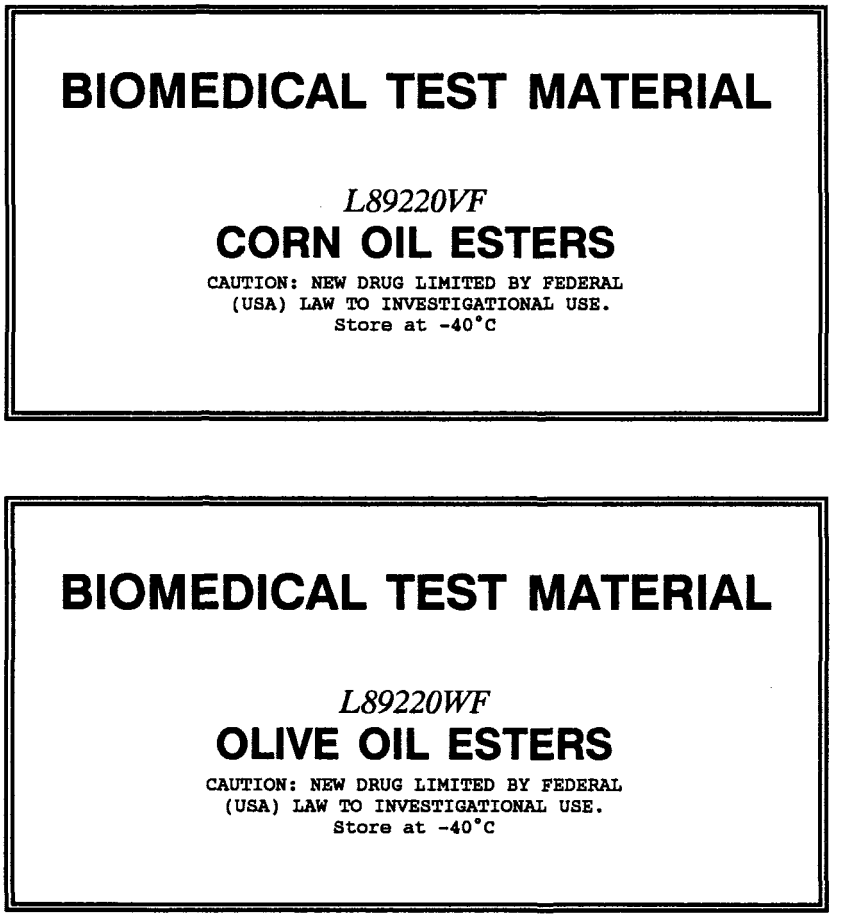
All of the plastic containers presently used for shipment of ethyl esters of vegetable oils are FDA approved for food use. High density polyethylene containers are used for shipment of the esters and are those supplied by Nalgene Corporation, Rochester, NY. The following stock numbers reflect the Nalgene amber, polyethylene food-grade containers:

2004-8125 (4 ml)
2004-9125 (4 ml)
2004-9025 (8 ml)
2004-9050 (15 ml)
2004-0001 (30 ml)
2004-0002 (60 ml)
2009-0004 (125 ml)
2009-0008 (250 ml)
2009-0016 (500 ml)
2009-0032 (1000 ml)
2009-0064 (2000 ml)

b. Labels

Labels are generated using computer fonts and a laser printer, photocopied onto self-adhesive labels and applied to the bottles. The label contains the following information: BIOMEDICAL TEST MATERIAL, LOT XXXXXXXX, Store at -40° C, "CAUTION: NEW DRUG LIMITED BY FEDERAL USE (USA) LAW TO INVESTIGATION USE". Example labels used on the bulk containers of ethyl esters of vegetable oils are shown in Figure 7-5.

FIGURE 7-5. EXAMPLE LABELS FOR BULK CONTAINERS OF ETHYL ESTERS OF VEGETABLE OILS.



3. STORAGE STABILITY of BULK ETHYL ESTERS OF VEGETABLE OILS

The storage stability of bulk ethyl esters of vegetable oils is currently being studied and will be reported at a later date. Sampling will be performed over two years, every six months. Parameters under study are delineated in Table 7-5. The integrity of the samples will be determined in terms of fatty acid data (qualitative and quantitative data obtained from TLC and GLC), peroxide values, tocopherol levels, and sensory analysis.

TABLE 7-5. STORAGE STABILITY OF BULK ETHYL ESTERS OF CORN OIL.

	MONTHS STORAGE, L89152VF				
ANALYSIS TYPE	**0**	**6**	**12***	**18**	**24**
ESTERS, %	87.8	88.5			
EPA, mg/g	<0.05	<0.05			
DHA, mg/g	<0.05	<0.05			
TOTAL n-3, mg/g	5.8	6.0			
16:0, mg/g	100.8	104.4			
18:1n-9, mg/g	224.7	231.9			
18:2n-6, mg/g	494.3	501.0			
a-TOCPOPHEROL, mg/g	1.1	1.6			
g-TOCOPHEROL, mg/g	2.0	2.2			
PEROXIDE VALUE, meq/kg	3.07	3.26			
SENSORY ANALYSIS:					
ODOR:		-			
TIO	3.66	-			
BUTTERY	0	-			
BEANY	0	-			
RANCID	0	-			
PAINTY	0.29	-			
OXIDIZED	0	-			
GRASSY	0.15	-			
FISHY	0	-			
BITTER	0	-			
SWEET	0.28	-			
FRUITY/MELON	0.63	-			
BURNT	0	-			
OLIVE	0.39	-			
ALCOHOL	0.56	-			
SOLVENT	0.88	-			
FLAVOR:					
TIF	3.4	-			
BUTTERY	0.25	-			
BEANY	0	-			
RANCID	0	-			
PAINTY	0.48	-			
OXIDIZED	0	-			
GRASSY	0.31	-			
FISHY	0	-			
BITTER	0	-			
SWEET	0.33	-			
FRUITY/MELON	0.59	-			
BURNT	0	-			
OLIVE	1.38	-			
CARDBOARD	0.81	-			
SOAPY	0.58	-			
SOLVENT	0.78	-			

* Data to be collected 6/90.

SECTION VIII. BACKGROUND AND SAFETY INFORMATION

Table of Tables

BACKGROUND AND SAFETY INFORMATION

1. INTRODUCTION

Menhaden provide the highest volume fishery in the United States, typically yielding over one million metric tons annually in recent years and approaching 50% of all U.S. commercial landings. The value of menhaden products manufactured in 1983, for instance, was $188 million. Virtually the entire catch is reduced to fish meal, oil and a smaller amount of fish solubles. Average U.S. menhaden oil production for the years 1983 and 1984 was about 170,935 metric tons (MT), representing some 98% of U.S. fish oil production. To set U.S. production in the framework of world production, it may be noted that world production of fish body oil grew from 269,000 MT in the period 1952 to 1.07 MMT in 1983.

There is considerable interest in menhaden oil as a potential nutritional supplement as well as interest in the potential pharmacologic action of specific components, the omega-3 (n-3) fatty acids. To foster a systematic approach to research in these areas the NIH/ADAMHA agreed to support n-3 research on a long term basis, provided that NOAA/NMFS would make available a specified group of high quality fish oil-derived test materials on a consistent long term basis.

In considering the issue of safety relative to the consumption of menhaden oil as a test material in the joint NIH/ADAMHA-NOAA/NMFS Fish Oil Test Materials Program, the following information is presented: description of the QA/QC Manual for the Test Materials Program, a description of the composition of menhaden oil showing it to be generally similar to other fish oils and edible vegetable and seed oils used in foods, and a description of the literature included in this file to support the safety of menhaden oil as a research material.

2. QUALITY ASSURANCE/QUALITY CONTROL

A complete manual of the methods currently used to characterize the chemical composition and the oxidative deterioration of fish oils is included as Appendix 2. The compilation of methods contained in this manual represents the standard methods utilized by the NMFS FISH OIL BIOMEDICAL TEST MATERIALS PROGRAM (BTMP) to conduct Quality Assurance and Quality Control. Many of the methods are AOAC or AOCS approved methods. In some cases where current technology far exceeds the official method, the Program has utilized the newer techniques.

The BTMP QA/QC manual contains nine sections including the general introduction. Each section of the manual deals with specific types of analyses: lipid characterization, sterols, fatty acid oxidation products, organics, metals, moisture, sensory attributes, and microbiological analyses.

3. COMPOSITION OF MENHADEN OIL

Menhaden oil is an animal oil produced from fish of the genus Brevoortia by cooking the fish and expelling the oil under pressure. The crude oil thus prepared is processed using adaptions of established methods for the preparation of other edible oils.

These products have no single chemical name since, like edible vegetable oils, they consist of complex mixtures of glycerides, fatty acids, unsaponifiables and phospholipids. The CAS registry number for menhaden oil is 8002-50-4. Menhaden oil consists mainly of a mixture of triglycerides of various long chain fatty acids with small amounts of mono- and diglycerides. The small amount of unsaponifiable material found in menhaden oil consists principally of squalene, sterols and pigments. Phospholipids generally occur at about the 0.1% level in menhaden oil.

The fatty acids that characterize menhaden oil are similar to those found in the various edible vegetable oils and animal fats, differing principally in the presence of polyunsaturated fatty acids with five and six double bonds which are unique to fish oils (Table 8-1). A complete characterization of the refined menhaden oil used to prepare test materials (both the soft gelatin capsules and bulk oils) was presented in Sections III and IV of this file entitled Processing and Chemical Composition Information for Test Materials produced by the BTM Program.

The fatty acid composition of the edible portions of finfish was reviewed by Exler and Weihrauch (1976). The data were obtained from many species and from different fish portions depending upon the species. An excerpt of these data are presented in Table 8-2; the data are recalculated as a percentage of the oil and include the fatty acid composition for 19 species of fish with profiles broadly comparable to that of menhaden oil.

Table 8-3 presents a comparison of the ranges of fatty acid composition for the selected 19 species of finfish with the fatty acid composition of oils obtained from three sources, menhaden, cod liver and sardine/pilchard. It can be seen that the values for menhaden oil generally correspond to the pattern found in widely consumed food fish. The range of values for 22:1 and 20:5 in sardine and pilchard oils are a little higher than in other food fishes, although they follow the same general pattern. The higher molecular weight fatty acids, characteristic of fish, show good agreement. Cod liver oil differs somewhat from menhaden oil with respect to several fatty acids: C14 and C16 fatty acids are lower in cod liver oil; 18:1 is about double the level found in menhaden oil; 22:1 is relatively high in cod liver oil; 20:5 is higher in menhaden oil and 22:6 is higher in cod liver oil.

TABLE 8-1. A COMPARISON OF FATTY ACID CONTENT OF MENHADEN OIL WITH SOME COMMON OILS AND FATS (g/100g).

Fatty Acid	Menhaden	Soybean	Peanut	Cotton Seed	Butter	Lard
4:0	-	-	-	-	3.2	-
6:0	-	-	-	-	1.9	-
8:0	-	-	-	-	1.1	-
10:0	-	-	-	-	2.5	0.1
12:0	-	-	-	-	2.8	0.5
14:0	9.0	0.2	0.1	0.8	10.1	1.4
16:0	19.0	10.7	9.5	22.0	26.3	23.7
18:0	3.8	3.9	2.3	2.3	12.1	13.0
16:1	13.3	0.3	-	0.8	2.3	2.6
18:1	15.5	22.8	45.6	18.1	25.1	40.9
22:1	0.7	-	-	-	-	-
18:2	2.0	50.8	31.0	50.3	2.3	10.0
18:3	1.0	6.8	-	0.4	1.4	1.4
20:5	12.5	-	-	-	-	-
22:6	7.9	-	-	-	-	-

TABLE 8-2. FATTY ACID CONTENT OF SEVERAL FINFISH SPECIES[1].

NAME	14:0	16:0	16:1	18:1	22:1[2]	20:5	22:6	(3)
Bass, striped *Morone saxatilis*	1.9	16.7	6.2	21.4	<0.2	8.1	22.4	F
Cod, Atlantic *Gadus morhua*	0.7	15.1	1.4	8.2	<0.7	11.0	20.5	F
Cod, Pacific *Gadus macrocephalus*	0.8	10.9	3.1	9.4	0.2	10.9	18.8	F
Flounder, yellowtail *Limanda ferruginea*	4.2	13.3	5.8	9.2	<0.4	9.2	9.2	F
Haddock *Melanogrammus aeglefinus*	1.5	12.1	3.0	9.1	<0.8	7.5	15.2	F
Hake, Pacific *Merluccius productus*	3.3	11.3	6.0	10.0	<0.3	14.0	14.7	F
Hake, silver (whiting) *Merluccius bilinearis*	4.5	13.4	7.6	15.0	<0.5	8.2	11.3	F
Halibut, Atlantic *Hippoglossus hippoglossus*	0.9	11.8	1.8	9.1	<1.8	9.1	27.3	F
Mullet, striped *Mugil cephalus*	10.5	12.0	13.4	9.5	0.5	8.5	6.6	E
Ocean perch *Sebastes marinus*	4.0	10.8	6.0	17.6	3.2	8.0	7.2	E
Pilchard *Sardina pilchardus*	6.0	14.0	8.5	12.4	2.1	12.7	17.9	F
Pilchard, African *Sardinops ocellata*	6.8	22.4	5.3	15.0	<0.1	18.2	7.4	F
Pollock *Pollachius virens*	0.5	8.0	1.0	6.0	1.0	7.0	33.0	E
Salmon, chinook *Oncorhynchus tshawytscha*	3.4	15.2	8.3	26.5	3.3	7.6	5.4	F
Salmon, pink *Oncorhynchus gorbuscha*	3.1	9.2	4.6	16.0	3.3	12.3	17.1	F
Salmon, sockeye *Oncorhynchus nerka*	3.5	13.5	6.3	10.6	0.1	14.6	19.1	F
Tuna, albacore *Thunnus alalunga*	2.8	20.0	4.9	17.5	1.5	7.9	21.2	W
Tuna, bluefin *Thunnus thynnus*	3.0	17.0	3.4	19.4	3.4	5.9	18.7	F
Tuna, skipjack *Euthynnus pelamis*	3.3	22.2	3.3	14.4	<0.6	6.7	12.2	F

1/ Values are % of fat, calculated from g/100g edible portion and total lipid figures tabulated by Exler and Weihrauch (1976, Table 1).

2/ Total isomers.

3/ The portion of the fish used to determine the fatty acid composition; fillet (F), white meat (W) or eviscerated (E).

TABLE 8-3. A COMPARISON OF THE PRINCIPAL FATTY ACIDS OF MENHADEN, SARDINE AND COD LIVER OILS WITH THE RANGE FOR FOOD FISH.

FATTY ACID	MENHADEN OIL[1]	RANGE FOR FOOD FISH[1]	RANGE FOR SARDINE & PILCHARD OILS[1]	COD LIVER OIL[2] MEAN	COD LIVER OIL[2] RANGE
14:0	8.9	0.5 - 10.5	6.6 - 7.8	3.2	1.5 - 6.8
16:0	19.2	8.0 - 22.4	15.5 - 17.8	13.4	11.0 - 18.8
16:1	11.4	1.0 - 13.4	6.0 - 9.5	9.6	6.8 - 11.9
18:1	10.6	6.0 - 26.5	9.2 - 17.3	23.3	17.1 - 31.6
22:1	0.6[3]	0.1 - 3.4	3.1 - 7.8	5.5	0.8 - 13.1
20:5	14.0	5.9 - 18.2	9.6 - 19.3	11.2	5.9 - 15.1
22:6	8.6	5.4 - 27.3	6.4 - 13.0	12.5	7.6 - 19.2

1/ Data from Ackman, 1982, Ackman, 1980a, 1980b, Exler and Weihrauch, 1976, Joseph, 1985.

2/ Data from Jangaard et al. (1967); means and ranges of 34 samples.

3/ Of the three 22:1 isomers included (n-11, n-9 and n-7) erucic acid (n-9) accounts for <0.2%.

4. SAFETY OF REFINED MENHADEN OIL AS A BIOMEDICAL TEST MATERIAL

The safety of refined menhaden oil as a test material for n-3 research was considered by an international conference `Health Effects of Polyunsaturated Fatty Acids in Seafoods' (Simopoulos et al., 1986) held in Washington, DC in June 1985. The conference was convened to review research data on the health effects of PUFA in seafood and to develop a research agenda to determine the spectrum of the health effects of PUFA of seafood origin in the American diet. The summary of the conference and recommended research agenda (see Simopoulos et al., 1986, Part I) presents both a historical perspective on fish oil and health effects in humans, as well as a definitive set of conclusions on the action of fish oils (or specifically n-3 PUFA) in several research arenas: eicosanoid formation; thrombosis and atherosclerosis; lipoproteins and atherosclerosis; immunology and inflammation; and DHA - membrane function and metabolism. A review of Part I indicates that there is no known toxicity from the consumption of elevated levels of n-3 fatty acids in the diet. The participants of one session (immunology and inflammation) summarized this by the following statement..."With regard to studies on humans, it was clear that to date there is no appreciable evidence of toxicity from the attempt to intervene in pathobiologic states, or from the administration to normal individuals of substantial doses of eicosapentaenoic acid (EPA)-enriched preparations in the form of MaxEPA or esterified EPA"...

The participants of the session on eicosanoid formation... "expressed an acceptance that the current evidence qualifies the omega-3 fatty acids as `essential' nutrients for which a requirement can be demonstrated"... "There was a belief that adults may successfully moderate their platelet function with 2g EPA per day"...The participants also suggested that... "The possible future use of concentrated preparations of omega-3 fatty acids in selected therapeutic treatments (as distinct from daily use to prevent disorders)" was felt to depend on further clinical studies that would have defined clinical and biochemical criteria. The BTMP was conceived to provide support to carefully planned clinical trials designed to add to the existing knowledge on the role of n-3 fatty acids.

The participants of the session on thrombosis and atherosclerosis concluded that it was time for limited, carefully controlled clinical studies and stated that the ..."existing knowledge is not sufficient to recommend large randomized population studies. In selected clinical disorders with accelerated thrombotic and atherosclerotic disease, however, trials with omega-3 fatty acids are indicated"... The BTMP, as was stated earlier, was conceived to provide support to carefully planned clinical trials designed to add to the existing knowledge on the role of n-3 fatty acids in coronary artery disease.

The participants of the session on DHA - membrane function and metabolism discussed numerous areas of research but provided the following expressions of caution..."in relation to peroxides and aldehydes that may be inadvertently fed, since these polyunsaturates are very susceptible to autoxidation"... "possible vitamin E depletion"... may occur,... " which can result from high levels of dietary polyunsaturates. The need for vitamin E supplements along with varying doses of fish oil therefore requires study". The BTMP addresses

these concerns directly and provides test materials that are carefully and extensively tested for quality. This information is provided to the researcher at the time of initial interview, is shipped with the test materials, and is updated on a routine basis. In addition, all test materials are continuously being monitored for long-term storage stability; these data have been provided in Section III of this DMF.

In response to the conference recommendations, NIH/ADAMHA (8 institutes) issued a joint program announcement inviting grant applications in the area of n-3 research. The need for test materials was enunciated by all session chairmen in this conference; in response the BTMP was initiated.

The conference published a proceedings (Simopoulos et al., 1986) which carefully detailed the then current research in the five working session areas. These proceedings are included in the DMF for refined menhaden oil to serve as current reference material.

The recently held AOCS Short Course on Polyunsaturated Acids and Eicosanoids (May 14-17, 1987 in Biloxi, MS) provides an up to date summary of the most current research in the area of n-3 fatty acids. The proceedings were published recently (Lands, 1987) and provide information on polyunsaturated fatty acids in the areas of platelet function (Session I), plasma lipid levels (Session II), cellular events of inflammatory processes (Session III), membrane turnover (Session IV), cancer (Session V), and development and role of n-3 acids in neural events (Session VI). Of the 35 papers presented, 13 were directly related to human studies and none of the 35 discussed the question of potential toxicity to humans of either the triglyceride form or the purified n-3 PUFAs. In fact, the vast majority of the papers suggested a protective effect or beneficial role for fish oil. A summary listing of these papers is provided in Table 8-4 and a copy of the proceedings is provided with other materials for the DMF.

A review of the literature for information on the consumption or exposure of a variety of species to menhaden oil is summarized in Tables 8-5 and 8-6. There are a total of 22 publications which reported the investigation of medium to long-term exposure to dietary fish oil in some form; 19 deal directly with menhaden oil. Most studies report beneficial decreases in selected parameters, while several reported a protective effect against an induced-disease state. It is important to differentiate between menhaden oil and other forms of fish oil such as capelin oil and cod liver oil. Capelin oil contains significant quantities of 22:1 isomers (15-18%); 22:1n11 accounts for most while erucic acid (22:1n9) accounts for nearly 2% of the total 22:1. Menhaden oil contains <0.2% erucic acid and less than 1% total 22:1. These levels are significant due to the observation that in some species, elevated erucic acid-containing oils (>2%) used as the entire source of dietary lipid can cause heart lipidosis. Cod liver oil contains significant levels of Vitamins A and D which have their own biologic activity and may cause effects incorrectly attributed to n-3 PUFAs. Long-term studies with pigs, rats, cats, and chickens indicated that no significant negative health effect could be attributed solely to menhaden oil, although with a high intake of unsaturated fat (eg. safflower oil, corn oil) there seemed to be some tendency toward decreased life-span in rats and mice (Harman, 1971). Numerous reports of the

beneficial protection by menhaden oil to consumers have been made, especially as a result of experiments studying induced disease states: lowering serum triglycerides in hyperlipidemic patients (Levine et al., 1984); a decrease in total plasma lipids in mild-diabetic patients (Kinsell et al., 1961); an increased platelet life-span and reduced platelet aggregation in atherogenic primates (Ward and Clarkson, 1984; 1985); reduction in myocardial damage in dogs with induced myocardial infarctions (Culp et al., 1980); protection for cats with induced cerebral infarctions (Black et al., 1979); a decrease in kidney arachidonic acid levels associated with lowered kidney damage in rats (Schoene et al., 1981); a decrease in blood pressure in spontaneously hypertensive (SHR), SHR/stroke prone, and normotensive rats (Schoene and Fiore, 1981); protection against renal disease (autoimmune nephritis) with prolonged survival in mice (Prickett et al., 1981; 1983); and, a decrease in serum cholesterol in chicks fed a hypercholesterolemic diet (Kahn et al., 1963a,b).

TABLE 8-4. A SUMMARY OF THE BILOXI WORKSHOP.

STUDY	TYPE STUDY, SUBJECT	FUNCTION STUDIED	TOX	COMMENTS
Session I. PLATELET FUNCTION:				
1	epidemiology	CHD	N	Greenland
2	clinical, epidemiology	blood parameters, CHD/EPA	N	Japan
3	dietary habits/epidem.	platelets/CHD	N	Norway
4	animal model	CLO, atherosclerosis	N	Pig, 8 mos.
5	human, FO supplement	blood pressure	N	50 cc/d, 3 mo, no blood press change, increase urinary PG.
6	human, diet modifications	blood functions.	N	1 yr, decrease saturated fat intake.
7	rat, FO supplement	heart functions.	N	12-15 mo.
Session II. PLASMA LIPID LEVELS:				
8	monkey, FO diet	atherosclerosis	N	8 & 52 wk; prevented atherosclerosis.
9	human, FO suppl	hemostatic functions	N	2 & 6 wk; normal & insulin dependent diabetics.
10	human, FO suppl	platelet phospholipids	N	22 & 42 d; 3.6g EPA & 2.4g DHA.
11	rat, FO diet	lipoprotein, cholesterol, hepatic enzymes	-	noted a reduction in LDL receptor status
Session III. CELLULAR EVENTS, INFLAMMATORY PROCESSES:				
12	mice, FO diet	arthritis, incidence & severity	N	5-15wk.
13	rat, FO gavage	CsA nephrotoxicity	-	14d + 14d w/CsA.
14	human, FO supplement	5-lipoxygenase pathway	N	6 wk; 3.2g EPA & 2.2g DHA/d.
15	rat, CLO & SNO diet	nephrotoxicity model	-	6 wk; prevent loss of renal function.
16	blood cell culture, natural killer cell (NK)	cytotoxicity	-	Emulsions of EPA- & DHA-TG, depress of NK activity.
17	human, EPA & DHA-EE suppl (caps)	neutrophil phospholipids	N	3.6g EPA or DHA/d, 2-4 wk;lipid composition altered; observed decrease in inflammatory responses.
18	murine autoimmune disease, FO diet	inflammation	N	Benefit to rheumatoid arthritis sufferers, FO reduced mortality & severity glomerulonephritis.
19	human, FO supplement, double blind, crossover study	rheumatoid arthritis, leukotrienes	N	14 wk-4wk-14wk-4wk, 2.7g EPA, 1.8g DHA; benefit when on FO; decrease in neutrophil leukotrienes on FO & correlates w/ decrease tender joints.

TABLE 8-4. Continued.

STUDY	TYPE STUDY, SUBJECT	FUNCTION STUDIED	TOX	COMMENTS
Session IV. MEMBRANE TURNOVER:				
20	rat, membrane composition	phospholipids-PC,PE.	-	Summary information on rat liver, rbc, hepatocyte,
21	cell culture	phospholipid classification	-	Structure analysis; arachidonate phospholipids.
22	human rbc's, rat liver & rbc.	distribution of n-3's in plasma membranes.	-	ETOH effects on DHA metabolism.
23	rat, FO & 18:3n-3 diet; 57% of fatty acids, n-3; 12 wk.	linolenic acid effect on AA metabolism	N	Summary of metabolites of 18:3n-3; less 5-HEPE formed w/18:3, FO n-3 more effective in suppressing 20:4n-6.
24	human, diet w/no EPA or AA; 2 wk.	plasma cholesterol esters.	N	Incorporate a-LNA into plasma cholesterol esters & transformed to EPA; decrease incorporation by linoleic.
Session V. CANCER:				
25	Review n-3 fatty acids and cancer.		-	Hypothesis re: n-3 as chemopreventive or anti-tumor.
26	Review dietary fat & colon cancer;		-	Although CO, SFO,lard ^colon tumors, FO, COCO, OO did not.
27	rat; FO,CO diet(20% total fat, ratio); 120d	preneoplastic development in rat pancreas	-	CO/FO-0/20 to 20/0; significant decrease in nodules w/FO; increase n-3/n-6 inhibits preneoplastic response.
28	rat; hi-fat 23% & lo- 5% diets;	mammary tumor promotion;	-	SFO,CO,OO,COCO studied; tumor incidence highest in HF diet polyunsaturated, HF monounsaturated lo incidence (ie. OO).
29	human, epidemiology: 121,700 reg.nurses; ages 30-55; 1976-1984 survey; 32-44% fat consumed	breast cancer	-	Moderate alteration of fat & fish intake no effect on incidence of breast cancer tho may have on CHD.
30	rat; linoleate-0.5 -11.5 % w/ total fat 20% (CO+COCO)	tumor incidence & yield; DMBA-induced tumor model.	N	EFA required for max expression of mammary neoplasia; mode of action of PUFA not understood.
31	rat, 5 diets- CO & MO 20/0 to 0/20	mammary tumor; latent period	N	Observed increase latent period w/ MO; increase EPA in tumor membrane in MO diet.

TABLE 8-4. Continued.

STUDY	TYPE STUDY, SUBJECT	FUNCTION STUDIED	TOX	COMMENTS
Session VI. DEVELOPMENT AND ROLE OF n-3 IN NEURAL EVENTS:				
32	Human epidem.	duration of pregnancy & intake of marine fat.	-	120 pregnancies, maternal blood FA, TL & PE; birthweight, gestation age, unaffected; birthlength obsev + assoc.
33	Review of membrane & EFA metabolism; compartmentalization of LA & AA; metabolism of a-LNA; conversion a-LNA to DHA; metabolic pool for PG synthesis.		-	Brain development may require a-LNA.
34	rat, hypertensive, 5% oil diet (SFO, perilla + rat chow)	retinal activity: brightness discrimination, ERG measurement.	N	Correct response ratio: perilla>normal diet group>SFO group. Pulmonary tumor metastasis: perilla<<normal diet=SFO.
35	rhesus monkey: pregnant females & infants; diet deficient n-3 (SFO), control (SO)	role of n-3 in development of retina & nervous system	N	Observe depletion of DHA (80-90%) plasma & tissues, associated with 3 abnormalities in visual function; conclusion, n-3 FA are essential.

Abbreviations

Oils: CO-corn, COCO-coconut, CLO-cod liver, FO-fish, MO-menhaden, SO-soybean, SFO-safflower, SNO-sunflower.

Other: AA-arachidonic acid, a-LNA-alpha linolenic acid, CHD-coronary heart disease, EA-erucic acid, EFA-essential fatty acid, FA-fatty acid, LA-linoleic, PE-phosphoethanolamine, PG-prostaglandin, 'oil'-EE-ethyl ester of an oil, TL-total lipid, TG-triglyceride.

TABLE 8-5. SUMMARY INFORMATION ON THE EXPOSURE OF MEDIUM TO LARGE MAMMALS TO MENHADEN OIL (fourteen publications attached).

Author(s)	Date	Study length	MO dietary level	Negative effects	Comments
HUMAN STUDIES:					
Ahrens et al.	1959	21-22wk	40%cal	none	No vit E supplement, no symptoms; creatin not elevated.
Levine et al.	1984	3wk	60 ml/d	none reported	Hyperlipidemic patient; decrease serum-TG, no change serum-cholesterol.
Kinsell et al.	1961	80d	10-45%cal	none other than those expected based on patient hist.	MO-EE = 'Escambia Oil' (~70% n-3); observed decrease total plasma-lipids.
Lands	1987	29d	15%cal	none reported	decrease in platelet aggregation and thromboxane production.
PRIMATE STUDIES:					
Ward & Clarkson	1984	8 wk	not given	none reported; platelet life-span increased.	FO prolonged bleeding time; inhibited platelet aggregation; increase n-3 in platelet membranes.
Ward & Clarkson	1985	8wk	20%cal	none reported; platelet life-span increased.	Animals fed atherogenic diets for 6 mo; observed prolonged bleeding times & reduced platelet aggregation.
Parks & Bullock	1986	8mo	11%	none reported.	studied changes in LDL size, composition and melting temperature.
Parks & Bullock	1987	8mo	42%cal	none reported.	increase in n-3 cholesterol esters; these esters accumulate in greater numbers in LDL to create enlarged particles.

TABLE 8-5. Continued.

Author(s)	Date	Study length	MO dietary level	Negative effects	Comments
PORCINE STUDIES:					
Barlow & Pike	1977	6-12wk	1-10%	none	Oxidized oil or meal used in diet.
Opstvedt et al.	1979	22d	none	all diets -> diarrhea.; heart lipidosis.	21% capelin oil; erucic acid cause; <2% EA in diet had no effect.
Opstvedt & Pettersen	1981	1.5-2.5y	15%	none observed.	No growth or organ abnormalities; pups a-OK.
Svaar et al.	1980	1y	none	minor heart lesions.	42%cal capelin oil.
CANINE STUDIES:					
Culp et al.	1980	36-45d	25%cal	none; FO reduced myocardial damage.	Experimental myocardial infarction.
FELINE STUDIES:					
Black et al.	1979	18-24d	8%cal	none; beneficial- protection vs cerebral infarction.	FO may avoid 'narrowing of vascular channels via PG E2 & F2a'.

Abbreviations

Oils: CO-corn, COCO-coconut, CLO-cod liver, FO-fish, MO-menhaden, SO-soybean, SFO-safflower, SNO-sunflower.

Other: AA-arachidonic acid, a-LNA-alpha linolenic acid, CHD-coronary heart disease, EA-erucic acid, EFA-essential fatty acid, FA-fatty acid, LA-linoleic, PE-phosphoethanolamine, PG-prostaglandin, 'oil'-EE-ethyl ester of an oil, TL-total lipid, TG-triglyceride.

TABLE 8-6. SUMMARY INFORMATION ON THE EXPOSURE OF RODENT AND FOWL SPECIES TO MENHADEN OIL (thirteen studies attached).

Author(s)	Date	Study length	MO dietary level	Negative effects	Comments
RAT STUDIES:					
Privett & Cortesi	1972	20wk	10%suppl	severely oxidized diets led to slow weight gain; lack of vit E led to hemolysis.	Used Vit E acetate; FA analysis of MO diet suspect; looked for peroxides in tissues; no indication if lack of growth related to lower food intake.
Privett et al.	1965	2,4,6mo	10%suppl	no mention.	Lipid class and TG composition in tissues; beta-FA for liver and epididymis. TG; no observation of 22:6.
Harman	1971	life span	5,10,20%wt	increased unsaturation of oil -> decreased life span;	Tumor incidence no significant difference; autopsy: mammary carcinoma, lung amyloidosis, lung carcinoma, myocardial fibrosis observed in all groups, no significance associated.
Kurata & Privett	1980	36,57,77, 100wk	5,20%wt, 3:7 CO:MO mix	no mention.	Time didn't influence microsome lipid compos.; FA compos. Of diet but not % has profound effect on liver FA compos.
Schoene et al.	1981	22wk	5%wt, 1:4 CO:MO mix	no mention.	Kidney tiss produced PGE2 and PGF2a: MO<CO. AA levels were decr on MO diet.
Schoene & Fiore	1981	22wk	5%wt, 1:4 CO:MO mix	no mention	Beneficial effect of MO on blood pressure in 3 strains of rat (SHR, SHR/SP, WKY).

TABLE 8-6. Continued.

Author(s)	Date	Study length	MO dietary level	Negative effects	Comments
DeSchrijer & Privett	1982	33wk	complex mixture of SFO+HCO+MO; suppl 5-10% MO.		Linoleic defic in some diets; in LA sufficient animals lower rate of AA synthesis from LA when on MO diets. MO groups gained more weight due to greater intake.
Hornstra	1982	8wk	none	none observed; potential hazard due to enhancement of activated platelet factor VII.	CLO, 45%cal supplemented with 5%cal SNO. Observed reduction in platelet aggregation, prolonged bleeding time, changes in various PG metabolites.
MOUSE STUDIES:					
Harman	1971	life span	5,10,20%wt	increased unsaturation of oil -> decreased life span.	Tumor incidence no significant difference; autopsy: mammary carcinoma, lung amyloidosis, lung carcinoma, myocardial fibrosis observed in all grps, no significant association.
Prickett et al.	1981,	life span	25%wt	none observed.	MO diet protected vs renal disease & prolonged survival.
Prickett et al.	1983	life span	25%wt	none observed; glomerular change eliminated by MO diet	MO diet protected vs autoimmune nephritis; acts primarily to reduce inflammation.

TABLE 8-6. Continued.

Author(s)	Date	Study length	MO dietary level	Negative effects	Comments
FOWL STUDIES:					
Kahn et al.	1963a	7,22,29d (7,21,28,42,49,112d)	2%wt (0.125-2%wt,FO-EE)	none observed.	Decrease in serum cholesterol on FO diet; decrease more dramatic in chicks fed CLO-EE.
Kahn et al.	1963b	3,13mo	none; (0.5%wt CLO-EE)	none observed; authors state that continuous ingestion of 0.5% ethyl ester well tolerated.	Hypercholesterolemic diets; no significant difference between CLO-EE and controls: body & organ weights, SGO-T, spleen, brain, adrenal & liver cholesterol; differences were noted between 3 & 13 mo for aortic cholesterol as well as serum-cholesterol.

Abbreviations

Oils: CO-corn, COCO-coconut, CLO-cod liver, FO-fish, MO-menhaden, SO-soybean, SFO-safflower, SNO-sunflower.

Other: AA-arachidonic acid, a-LNA-alpha linolenic acid, CHD-coronary heart disease, EA-erucic acid, EFA-essential fatty acid, FA-fatty acid, LA-linoleic, PE-phosphoethanolamine, PG-prostaglandin, 'oil'-EE-ethyl ester of an oil, TL-total lipid, TG-triglyceride.

5. SAFETY OF OMEGA-3 ETHYL ESTERS

There is substantial evidence that n-3 fatty acids resident in fish oil in the triglyceride form are readily released during the digestive process and absorbed as fatty acids or in association with carriers. Evidence to support the contention that ethyl esters of n-3 fatty acids are also digested in a similar manner, prior to absorption, is available in the literature.

Dr. Norman Salem, Jr. has conducted studies in his laboratory at NIH/A-DAMHA to compare the absorbability of fish oil and ethyl ester concentrate from microencapsulated products with bulk products. During a ten day trial using rats and diets prepared from microencapsulated or bulk test materials similar fatty acyl distributions were noted in plasma, platelets, erythrocytes, liver, and brain tissues for both the ethyl ester and triglyceride forms of the test materials. In addition, Dr. William Connor (Dept. of Medicine of the Oregon Health Sciences University) presented data to the NIH/NCC Fish Oil Test Materials Committee indicating similar absorption of the fatty acids of fish oil as hydrolyzed from either the ethyl ester or triglyceride forms. The study involved the consumption and digestive hydrolysis of these materials consumed along with breakfast by human subjects.

The direct absorption of ethyl esters of n-3 fatty acids does not appear to occur to any substantial extent. Studies conducted in Dr. John Vanderveen's laboratories at the FDA indicate that there is no detectable absorption of ethyl esters of EPA and DHA (<4ng/ml of lymph) into the lymph system of canulated rats (Attachment 6, Summary of FDA Non-Clinical Laboratory Study-Final Report). An important conclusion of three other investigations was that ethyl ester forms of n-3 fatty acids are not absorbed into the mammalian blood stream to any significant extent. Terano et al. (Atherosclerosis 46:321-331, 1983) states on page 326 that "no ethyl ester form of EPA was detected in plasma" after ethyl ester of EPA was given to humans. In the publications by Kobatake, et al. (J Nutr Sci Vitaminol 30:357-372, 1984) weight gain and organ weights of rats fed omega-3 ethyl esters were not significantly changed. Harris et al. (Am J Clin Nutr, 48:992-997, 1988) demonstrated that triglyceride or methyl ester forms of omega-3 fatty acids produce the same effects on levels of plasma cholesterol, triglycerides, and lipoproteins.

These data substantiate the assumption that the safety and toxicology information provided above for menhaden oil should be applicable to the ethyl ester concentrate and to the more highly purified forms of omega-3 ethyl esters.

6. CONCLUSION

There is substantial evidence in the literature which supports the hypothesis that refined menhaden oil, and omega-3 ethyl esters obtained from this oil, may be safely used in human clinical research studies. The Biomedical Test Material Program's test materials are highly refined and thoroughly quality assured (See Sections III, IV, V, VI, & VIII and review the QA/QC Manual, Appendix 2). Each request for use of the test materials will be carefully scrutinized by the NIH/NCC FOTMA Committee; the researcher will have provided clear evidence of an internal review process at his institution as well as presentation of peer-reviewed and approved research hypotheses. Investigators planning to use the test materials in human studies will be required to complete the FDA's IND process. The researcher will receive test materials that have been produced under a rigorously quality controlled process (See Sections III, IV, V, VI, & VIII, and review Production Manual, Appendix 1). Each researcher will be provided complete quality assurance data for individual lots of test material (review Distribution Management Manual, Appendix 3).

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ATTACHMENT 5

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
BUREAU OF FOODS – NONCLINICAL LABORATORY STUDY
FINAL REPORT

Form Approved
OMB No. 0910-0119
Use of this form is prohibited after April 30, 1983.

NOTE. Form FDA 3224 must be completed at the conclusion of each study subject to the Good Laboratory Practice Regulations (21 CFR 58).

1. REPORT TITLE

Absorption of eicosapentaenoic and docosahexaenoic acids from purified ethyl ester concentrates or in menhaden oil by adult male Wistar rats

2. FACILITY (Name and address)
CFSAN:ONFS:DON:ENB:NTS
200 C Street, S.W.
Washington, DC 20204

3. STUDY DATES (As defined in protocol)	a. DATE STUDY INITIATED	b. DATE STUDY COMPLETED
	7-12-88	8-12-88

4. NAME OF STUDY DIRECTOR
Marla Reicks, Ph.D.

5a. BFO NO.
232-N

CONTRACT NO.
N/A

c. NAME OF CONTRACTOR
N/A

d. NAME OF PRINCIPAL INVESTIGATOR
N/A

7. OTHER AUTHORS AND STUDY PERSONNEL (Include names and titles)
James Hoadley, Ph.D, Staff Fellow
Jeanne I. Rader, Ph.D., Supervisory Research Chemist
M.R.S. Fox, Ph.D., Supervisory Research Chemist
S. Subramaniam, Biologist
Catherine Paul, Biological Laboratory Technician

8. STUDY OBJECTIVE (If additional space is required, use 8½ x 11" sheet)

The objective of this study was to compare the absorption kinetics of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in adult male Wistar rats when administered as purified ethyl ester concentrates or as natural components of menhaden oil. Lymph fluid was collected from a thoracic duct cannula at 4, 8 and 24 hours after administration of EPA and DHA via an indwelling duodenal catheter. EPA and DHA were administered as lipid emulsions containing the following lipid type: 1) ethyl ester concentrate, 2) menhaden oil, 3) EPA and DHA as free acids, or 4) ethyl ester concentrate plus olive oil. Absorption of EPA and DHA was determined by analyzing the fatty acid composition of the lymph fluid by capillary gas chromatography.

9 INTRODUCTION (If additional space is required use 8½ x 11" sheet.)

Natural fish oil products can be purified and esterified to form stable ethyl esters. These purified ethyl ester concentrates (80% ethyl esters of EPA and DHA) are commercially available as a 1.4 - 1.6:1 mixture of EPA and DHA (National Marine Fisheries Service, Charleston, NC). Although the ethyl ester forms of EPA and DHA have been used in animal and human experimentation for the past 25 years, little is known concerning their absorption and metabolism.

In this study, male adult Wistar rats were given EPA and DHA at approximately the same levels in one of four lipid emulsion preparations: 1) ethyl ester concentrate, 2) menhaden oil, 3) EPA and DHA free acids, or 4) ethyl ester concentrate plus olive oil. The lipid emulsion was given via an indwelling duodenal catheter and lymph fluid was collected via a thoracic duct cannula. Body weight was recorded weekly until all rats were cannulated, dosed and lymph collections completed. Lymph fluid lipid was extracted and fatty acid methyl esters were prepared and quantified by capillary gas chromatography.

10. SUMMARY (If additional space is required use 8½ x 11" sheet.)

At 4 hours after dosing a significantly higher percentage of EPA was recovered in the lymph in the group receiving EPA as the free fatty acid than in the groups receiving the ethyl ester concentrate with or without olive oil.

Combining the ethyl ester concentrate with olive oil did not change the percent EPA or DHA recovered in the lymph at any time period.

When recoveries for the 4, 8, and 24 hour collection periods were combined, the EPA in menhaden oil was recovered in a significantly higher amount than when the EPA was administered as the ethyl ester concentrate with or without olive oil.

At both 4 and 8 hours after dosing, the pattern of absorption and recovery of DHA was the same as that observed for EPA.

In the period from 8 - 24 hours, more DHA was recovered when the source was the ethyl ester concentrate than when it was the free fatty acid form.

When recoveries for the 4, 8, and 24 hour collection periods were combined, there were no statistically significant differences in the amount of DHA recovered in the lymph in any groups.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

CFSAN – NONCLINICAL LABORATORY STUDY
AMENDMENT TO FINAL REPORT

1. REPORT TITLE	2. BFQ OR CONTRACT NO.
Absorption of eicosapentaenoic and docosahexaenoic acids from purified ethyl ester concentrate or in menhaden oil in male Wistar rats	232-N

3. TYPE OF CHANGE (check one)	4. SECTION OF REPORT AMENDED
☐ CORRECTION ☒ ADDITION	16. Results

5. REASON FOR AMENDMENT

Additional results are reported on the thin layer chromatographic procedure (SOP MR192) used for the lipid extracts of the 4 hr lymph samples collected in Part A of the study.

6. DESCRIPTION OF CHANGE (If additional space is required use 8½ x 11" sheet)

The following results are added to Section 16 of the final report.

16. Results

Thin-layer chromatography of the 4-hr lymph samples

There were no bands observed on the thin layer plates which corresponded to the band produced by the ethyl ester standard for any rats in any treatment groups, indicating that there was no intact ethyl ester present at 4 hrs post-dosing.

GC chromatography

The area on the thin layer plates (0.25 ml lymph used for lipid extraction) which corresponded to the band produced by the ethyl ester standard for the rats in Group 1 (EE) was scraped and analyzed for fatty acid composition by gas chromatography. There were no EPA or DHA peaks detected, confirming the thin layer chromatography results that no intact ethyl ester was present in the lymph 4 hrs post-dosing. The detection level for EPA and DHA for the instrument and operating conditions used in this study is 1 ng fatty acid. This corresponds to the detection of a peak when 1 µl of a solution containing 1 µg fatty acid/ml iso-octane is injected into the gas chromatograph. Therefore, there is < 1 ng EPA or DHA/ 0.25 ml lymph.

7. SIGNATURE OF STUDY DIRECTOR	8. DATE
Marla Reecks	12-15-88

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