NOAA TECHNICAL MEMORANDUM NMFS SEFC - 243

BIOMEDICAL TEST MATERIALS PROGRAM:

DISTRIBUTION MANAGEMENT MANUAL

P. H. Fair

December 1989

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Center
Charleston Laboratory
P.O. Box 12607
Charleston, SC 29412-0607

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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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1.0 INTRODUCTION

1.1 BIOMEDICAL TEST MATERIAL PROGRAM

The epidemiological studies by Bang et al.[1] and Dyerberg[2], which associated the beneficial effects of omega-3 (n-3) polyunsaturated fatty acids (PUFA) with the potential prevention of coronary heart disease have stimulated numerous studies on the role of n-3 fatty acids. Seafood lipids may have a positive effect on preventing and/or ameliorating a number of disease states common in Western society. The physiological effects of increasing consumption of seafoods or fish has established beneficial effects on risk factors for coronary heart disease in a number of animal and human studies. Determining the scope of this potential, as well as any accompanying adverse consequences, will require additional clinical, biochemical, and physiological studies with high purity, quality-assured test materials of consistent composition that can be supplied to researchers over the period of time necessary to complete the research. The conference held in 1985 on "Health Effects of Polyunsaturated Fatty Acids in Seafoods"[3] concluded that the lack of adequate supplies of quality assured test materials of consistent composition and high purity was a significant limitation to exploring the research frontiers on preventing or ameliorating degenerative disease states.

In order to facilitate the evaluation of n-3 fatty acids in health and disease, an interagency program, the Fish Oil Biomedical Test Material Program, was established to respond to this need for reliable test materials which will be available over the period of years necessary to complete the research. The Biomedical Test Materials (BTM) Program was formally initiated in 1986 by the signing of a Memorandum of Understanding (MOU) between the National Institutes of Health (NIH), the Alcohol, Drug Abuse and Mental Health Administration (ADAMHA), and the National Oceanic and Atmospheric Administration (NOAA) of the Department of Commerce (DOC). Under the MOU it was agreed that the NIH, ADAMHA, and the National Marine Fisheries Service (NMFS) of NOAA would cooperate on, and provide support to, research activities related to the biological mechanism by which a seafood diet or the ingestion of fish oils may influence health and modulate a number of human processes. The NOAA/NMFS Charleston Laboratory is committed under this MOU to provide a long-term, consistent supply of quality-assured and quality-controlled test materials to researchers in order to facilitate the evaluation of the role of omega-3 fatty acids in health and disease states.

1.2 DISTRIBUTION MANAGEMENT

The aspects of packaging addressed in this manual represent those utilized by the NMFS Fish Oil Biomedical Test Materials Program. The manual contains six sections including the Introduction. Section 2 is concerned with the distribution management project and research requests, addressing the approval process, test material availability, communication/technical support, and coordination aspects. There are four sections that address a specific test material delivery form: bulk, soft-gel capsules, aerosol containers, and microcapsules. Each packaging method is described using the following guidelines: introduction, process, packaging, and stability.

The objective of the Distribution Management Project is to maintain the high quality of the Biomedical Test Materials (BTMs) during their packaging, storage, distribution and use by individual researchers and clinicians thus preserving the identity, purity, and safety of the test materials. The Distribution Management Project serves as a focal point for interaction between investigators and the BTM Program as well as investigating the development of various dosage delivery systems that facilitate research protocols. N-3 fatty acids and their derivatives are highly unsaturated, they are extremely susceptible to oxidative deterioration since oxidation reactions occur at the double bonds in the carbon chains of the fatty acids. Quality assurance information provided to the researcher provides quantitative data on the lipid composition, the presence of any oxidative products, and non-lipid components, including environmental contaminants. To assist in maintaining the quality of the test material, the Distribution Management Project in conjunction with the Quality Assurance Project subjects each type of test material to a formal stability study in the containers used in packaging. Stability studies are also conducted on proposed experimental protocols that may pose a concern over stability of the material prior to shipment of the test materials. Recommendations are then made on how to best store the test materials and on the optimum length of storage time. The chemistry of these high purity test materials (fish oils, and n-3 fatty acid esters) poses handling and delivery problems due to their markedly oxidative reactivity. Improper handling procedures may lead to oxidation of the PUFA and the use of oxidized PUFA can produce misleading results and may have health implications. Processing, preparation, and storage conditions of PUFA can result in the formation of peroxidic acids, hydroperoxides and their end products, polymers and oxidized sterols. When ingested, these oxidized components have caused several problems in experimental animals including hepatomegaly, steatitis, hemolytic anemia, and poor growth. It is essential that oxygen be kept from the test materials. Precautions are taken during the processing of the BTM at the NMFS Charleston Laboratory to assure that the BTM are protected from oxygen and possible contamination.

The objective of the Distribution Management Project in maintaining the high quality of the Biomedical Test Materials (BTMs) is accomplished by adhering to guidelines and the standards of the pharmaceutical industry. The Distribution Management Project uses FDAs Current Drug Good Manufacturing Practices (1978 Final Rules) as a guideline regarding testing and approval (or rejection) of components, product containers and packaging materials. The delivery method must ensure that the test materials are not degraded or adulterated before entering a biological system. The products involved in the various modes of packaging include: bulk and encapsulated vacuum-deodorized menhaden oil, encapsulated steam-deodorized menhaden oil, bulk and encapsulated n-3 ethyl ester concentrate, bulk and encapsulated ethyl esters of vegetable oils (corn oil, olive oil, safflower oil) and encapsulated vegetable oils (corn oil, olive oil, safflower oil). Purified eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) are available in bulk form.

The Distribution Management Project oversees the receipt of approved requests for test materials, liaison and technical support with the researchers and coordinates the production of the test materials for approved researchers. Several computerized data bases facilitate the storage and retrieval of information required to conduct these activities. In addition, the project is responsible for the development of various dosage delivery

systems that may facilitate specific research protocols. At the present time the test materials are available in bulk or in soft-gel capsules; other forms of packaging such as microencapsulation, aerosols, and edible whips are under investigation. The distribution of limited amounts of the test materials is on a gratis basis to approved researchers located within the United States; researchers applying from outside the U.S. incur shipping costs.

Technical Support: Personal interaction with the researchers by project staff provides a positive forum to discuss the development of the best protocols for the utilization of the test materials while maintaining their high quality. Information provided to the researcher by the Distribution Management Project includes: complete Quality Assurance (QA) information on the lot of material shipped; a QA Manual for the analysis of fish oil test materials; instructions on test material handling and storage, both in regard to animal diet preparation and the balancing of antioxidant levels in placebo oils purchased by the researcher. Other information deals with optional product forms that can be obtained by the researcher such as pressurized cans with metering valves and specially formulated edible whips. In addition, the researchers are advised on the feasibility of using a variety of flavoring agents as well as other considerations for double blind studies including proper placebos and controls for use in n-3 studies. Each of the test materials produced by the BTM Program is currently being subjected to formal stability studies in order to provide researchers with recommendations on specific storage conditions of the test materials and the length of time they can be expected to be stable.

Product Form: Bulk vacuum deodorized fish oil (VDFO) and ethyl ester concentrates of the VDFO are presently being distributed to a large number of researchers engaged in animal feeding studies. The VDFO and ethyl ester concentrates of VDFO are also available in a soft-gelatin encapsulated form and are being distributed to researchers engaged in human clinical studies. Commercial preparations of corn, olive, and safflower oils as well as their ethyl esters are available in the soft-gelatin encapsulated form for researchers to use in double blind studies in conjunction with the VDFO and ethyl ester concentrates of VDFO. Microencapsulation of the oil and ester may have potential application in animal studies as well as facilitating human use. A feasibility study has been conducted to ascertain the stability and efficacy of microencapsulated BTMs. Pressurized cans with metering valves may be a convenient method of storing and dispensing either the oil or the esters. This method also has the added ability of maintaining the high quality of the materials because of the constant pressure of a nitrogen atmosphere that is present in the can. Another possible product form is an edible whip foam contained in an aerosol can. Preliminary studies have been conducted on this product form with a commercial company, and the results appear promising.

2.0. BIOMEDICAL TEST MATERIALS

2.1 APPROVAL PROCESS

The availability of fish oil test materials is announced periodically in the National Institutes of Health (NIH) Guide for Grants and Contracts, as well as in journals and newsletters. The request process is initiated by the researcher who responds to the notice of availability or by requesting an application from the NIH Nutrition Coordinating Committee (NCC) Office. The researcher completes the forms specifying the particular type(s) and quantity of test material requested. Information is supplied regarding funding type and approval, research hypothesis, and description of research proposal. A statement of waiver of liability accompanies each request and must be signed by the investigator and the institutional office. If the researcher intends to use the materials in human research both an Investigative Review Board (IRB) No. from the investigator's institution and an Institutional New Drug (IND) approval from FDA is required. 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) has established a drug master file (DMF)[4] at the Food and Drug Administration which includes manufacturing, chemical composition and toxicological data relevant to these products. Investigators awarded materials may then reference this file in order to expedite their IND requests. Applications for omega-3 research materials for both human and animal studies are accepted and processed.

The system for the distribution of the Biomedical Test Material (BTM) produced by the Charleston Laboratory consists of the review and recommendation on the completed application by the Fish Oil Test Material Distribution Committee (FOTMDC), a subcommittee of the NIH/NCC. The FOTMDC is composed of NIH and other Federal scientists that are not involved in the application and use of these products. The distribution committee processes the applications received from investigators and advises the Department of Commerce (DOC)/National Oceanic and Atmospheric Administration (NOAA) 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. The present allocation of test materials is based on the following: a request of 1% or less of the yearly production of a test material may be awarded by the FOTMDC Chairman without board discussion; a request between 1-5% of the yearly production of a test material will be subject to review; and requests greater than 5% of the yearly production of a test material will be subject to negotiation for grant support of the BTM. To qualify to receive any amount of test materials described in the NIH Announcement the applicant must: 1) have a peer-reviewed project and 2) submit a correctly completed application form and a signed waiver of liability. Prioritization for receiving test materials will be based on funding for research projects which are: 1) NIH/ADAMHA funded 2) other government funded, 3) peer-reviewed, privately funded, and 4) NIH/ADAMHA approved, not funded and 5) other. Federal and state regulations on human subjects and animal research apply. In accordance with Federal regulations, an IND is required for the use of these test materials in human studies. The overall

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

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 advise 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 recommendations on experimental semipurified diets (Appendix 4).

2.2 AVAILABILITY OF BIOMEDICAL TEST MATERIALS

The Fish Oil Test Materials Program currently has available the following test materials.

* ***n-3 Ethyl Ester Concentrate*** - prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction and short-path distillation, containing approximately 80% n-3 fatty acid ethyl ester (44% EPA, 24% DHA, 10-12% other n-3 fatty acid ethyl esters), 3% C18 (other than n-3), 6% C16 and the remainder as other esters. It contains 0.2 mg/g tertiary butylhydroquinone (TBHQ) as antioxidant, 2 mg/g tocopherols and approximately 2 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' need.

* ***Placebo Ethyl Esters*** - may be prepared from any commercial oil by transesterification. Olive oil ester specifications are: approximately 70% oleic acid, 13% C16, and 15% C18 (<1% n-3) fatty acid ethyl esters. The preparation from corn oil ethyl esters contains approximately: 50% linoleic ethyl ester, 10% C16, and 15% other C18 (>1% n-3) fatty acid ethyl esters. Safflower oil ethyl ester contains approximately: 76-81% 18:2n6, 9-14% 18:1n9, 6-7% 16:0, and 1-4% 18:0. The placebo ethyl esters contain 0.2 mg/g TBHQ as antioxidant and 2 mg/g tocopherols. The preparations are available in 1 g soft-gel capsules (100 capsules/bottle) or packaged in bulk in quantities suitable to investigators' needs.

* ***Vacuum-Deodorized Menhaden Oil (VDFO)*** - prepared from menhaden oil that has been winterized and alkali refined and processed through a two-stage wiped-film vacuum-deodorizer to remove cholesterol, volatile oxidation products and any traces of organic contaminants. The oil contains approximately 30% n-3 fatty acids in the triglyceride from; 14% EPA, 8% DHA, 8% other n-3. It contains 0.2 mg/g TBHQ as antioxidant, 2 mg/g tocopherols and approximately 2 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 are considered.

* ***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% 18:2n-6, 26% 18:1n-9), olive (57% 18:1n-6, 17% 18:2n-6), safflower (80% 18:2n-6, 9% 18:1n-9). They are available in 1 g soft-gel capsules (100 capsules/bottle). Although vegetable oils are not supplied in bulk form, investigators may request analysis of antioxidant and tocopherol levels in vegetable oils that they purchase.

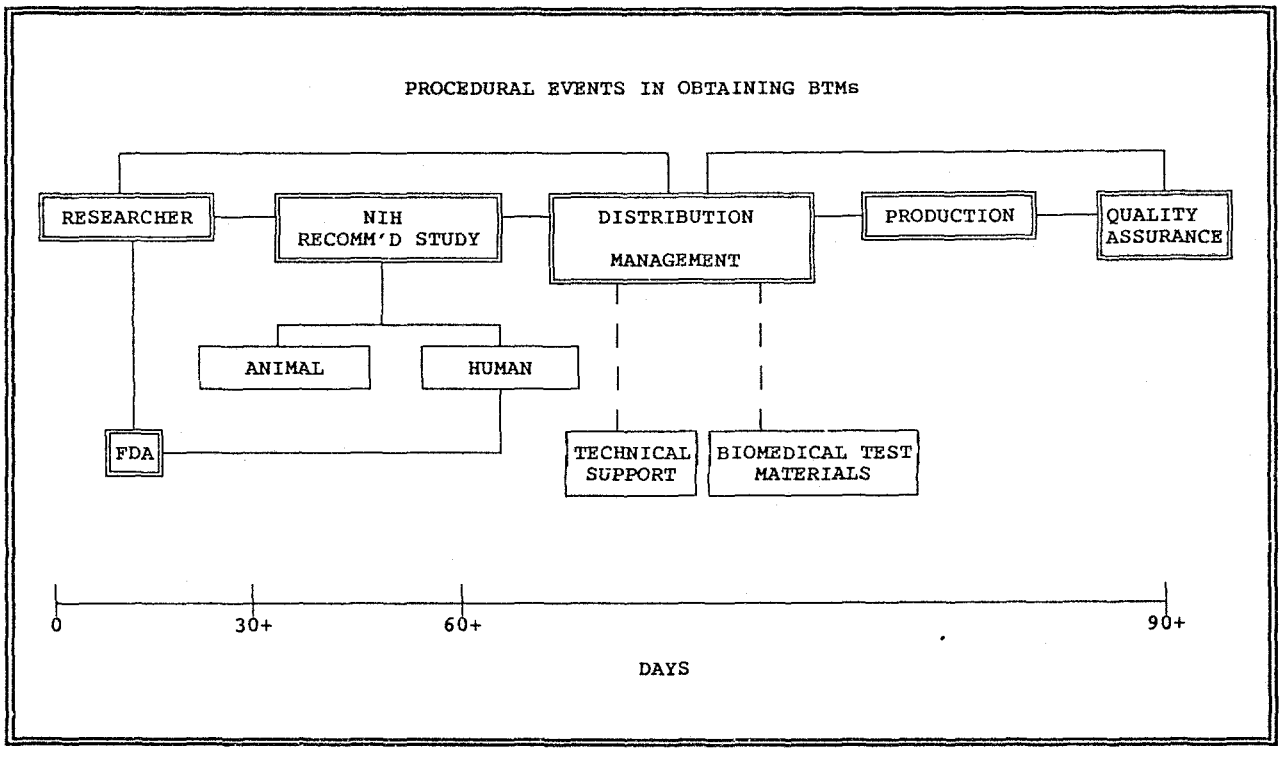
* ***EPA ethyl ester and DHA ethyl ester (>95% purity)*** - The ethyl ester of EPA and DHA is prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction and short-path distillation to yield an n-3 ethyl ester concentrate. The purified ethyl ester of EPA and DHA is attained by supercritical CO_2 extraction from the n-3 ethyl ester concentrate followed by high performance liquid chromatography. The EPA ethyl ester contains: >95% ethyl esters; of the ethyl esters EPA is 97%, other n-3's are <1%, n-6's are <1% and other fatty acids are <1% The DHA ethyl ester contains: >95% ethyl esters, of the ethyl esters DHA is 96%, other n-3's are <2%, n-6's are <1% and other fatty acids are <1% EPA and DHA are available in bulk form packaged in 1-5 g aliquots.

All products are prepared under a blanket of nitrogen and are supplied with detailed quality assurance data. Investigators may obtain further information and apply for available fish oil test materials for relevant studies by requesting an application from:

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

2.3 COMMUNICATION/TECHNICAL SUPPORT

The researcher is informed of the FOTMDC's decision via a letter from the BTM Program Manager at the Charleston Laboratory (NOAA/NMFS). The distribution management project maintains files on each researcher with copies of the application, signed liability statement, the letter from FDA assigning an IND number to the investigator, and all written correspondence. These same records are maintained at the FOTMP office. Telephone memos are also maintained in these files and are available to the FOTMP office upon request. A schematic diagram of the communication exchange appears below:



Communication between the researcher and the BTM Distribution Management Project is an important linkage in the BTM Program. Researchers may seek initial information on the program, test materials, or advice on the experimental protocol they will be using. Representatives from granting agencies also request information on the BTM and their availability. Approved researchers may seek information concerning analytical data, assistance with problems they may be experiencing or requesting additional materials. The Distribution Project Manager is authorized to approve additional allotments up to 20% of the total requested amount. Researchers requesting additional quantities of BTM exceeding 20% of the total approved amount of BTM will need to reapply to the FOTMDC for approval.

Each U.S. researcher approved for the requested amount of Biomedical Test Material (BTM) by the FOTMDC receives a telephone call soon after receipt of the approval letter. Telephone conversations provide a positive forum to discuss the development of the best protocol for the utilization of the test materials while maintaining their high quality. This interchange is an important component of the BTM Program. The initial conversation covers a diver-

sity 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 individual needs. Some researchers have significant experience in handling polyunsaturated fatty acids and do not require as much assistance as those just entering the n-3 arena. Technical support may be provided in the areas listed below. The information obtained from the application and the telephone conversation is documented on specially designed forms that are bound into a researcher request notebook. The conversation with the researcher covers a diversity of topics depending upon their research area and needs but some of the essential areas that are routinely discussed are described below:

1) Introduction and the Role of the Contact Person (liaison, support, coordination, information, etc.)

The initial portion of the conversation serves to establish the role of the contact person and the types of information and technical support that can be provided.

2) Research Protocol Discussion

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

It may be necessary to describe the types of BTM available and to provide updated information to the researcher on available products. If the use of the BTM in a particular circumstance is questionable, another more appropriate dosage form of the BTM is suggested. Verification is received on the number of experimental units (animal/human) and the dosage being given on a daily basis and the length of time for the experiment in order to quantify the total amount of requested material; included is an appropriate amount for spillage/waste in animal experiments.

b) Antioxidants

The researcher is informed on the typical amounts and types of antioxidants utilized in the production process of the BTM. If the use of antioxidants poses a problem with the experimental hypothesis, the VDFO can be obtained without antioxidants completely or with just TBHQ or tocopherols depending on the need. Researchers are urged to use the test materials with antioxidants if possible to enhance their stability during use. The fish oil ethyl ester concentrate may not be obtained without antioxidants since the production process incorporates the addition of antioxidants and there is greater concern over its stability. Information is provided on the balancing of antioxidants in control treatments to assist in the experimental design (Appendix 1), including the offer of analytical support for determining the concentration of endogenous antioxidants in the control/placebo treatment being used. If antioxidant analyses is desired, the researcher is advised to

send a 5 ml sample in an amber, glass or plastic container to the Charleston Laboratory. Analytical results of final concentration of antioxidants in the control treatment may also be verified upon submission of a sample after addition of antioxidants. Information is provided regarding the stability of the BTM in the various delivery systems.

c) Use of control/placebo treatment

Information is obtained as to whether the research protocol has the proper control for use in animal studies and human trials. The types of placebos available is discussed. The Charleston Laboratory will assist the researcher in double blind studies by flavoring the placebo with fish flavor to resemble the fish oil or n-3 ethyl ester concentrate or by using a flavoring agent on all treatments[5]. The process used in balancing the antioxidants in the fish oil and/or n-3 ethyl ester is explained to the researcher. The researcher must obtain the placebo oil of their choice (corn oil, olive oil, safflower oil, etc.) for use with the bulk vacuum-deodorized fish oil and the Charleston Laboratory will analyze the oil(s) for endogenous antioxidants. The researcher may then add the specified amount of antioxidants to balance those in the fish oil and the Charleston Laboratory will verify final antioxidant concentrations by analysis. Bulk placebo ethyl esters of corn oil, olive oil, and safflower oil are available with the antioxidant content similar to that of the n-3 ethyl ester concentrate. Soft-gel capsules of the following placebo oils are provided with appropriately balanced antioxidants: corn oil, safflower oil, olive oil, and their ethyl esters.

d) Length of study

The distribution project will set up a suitable delivery schedule depending on the availability and stability of the BTM and the research experimental protocol. Generally, shipment of <u>bulk</u> test materials should be limited to a 6 month time period for n-3 ethyl ester concentrate and a 12 month period for the VDFO. <u>Priority</u> is given to providing a continuing supply of test materials to studies that have been initiated using specified test materials.

e) Packaging requirement

The BTM is custom packaged in a manner consistent with 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 thereby reducing potential for oxidation. For instance, the BTM is custom packaged according to the quantity utilized in formulating the amount of diet at one time, or the amount of material to be used in gavage studies for a 1 week period. Most studies utilizing animals request bulk BTM for incorporation in the diet or for use in gavage studies. A few animal studies have utilized capsules, such as in the case with primates trained to consume capsules. Most researchers conducting studies with humans prefer the use of the BTM in soft-gel capsules which have excellent stability over a long time[6]. A simulated trial of taking home a bottle of bulk n-3 ethyl ester concentrate, opening 3 times a day at refrigerated temperatures revealed an 10 fold increase in peroxide values from 2 meq/kg to 21 meg/kg over a 7 day period. The opportunity for abuse is significantly high enough to warrant concern over this method of product handling. Caution is urged in the use of BTM in the bulk form for human use. The use of bulk BTM for human

studies should require daily aliquots to be made by the clinician in appropriate containers and optimum storage conditions for the duration of the experiment.

3) Human Studies

The use of BTM in human studies requires the acquisition of an IND number. The investigator is responsible for securing an IND number and complying with the monitoring and reporting requirements of the FDA. NIH/ADAMHA/DOC does not hold an IND for the BTMs. A Drug Master File (DMF) prepared by DOC and submitted by NIH/ADAMHA to the FDA 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. Researchers using VDFO are authorized to reference DMF number 7436; researchers using n-3 ethyl ester are authorized to reference DMF number 7779. Approval letters to researchers provide an explanation on the application process for an IND number and FDA forms 1571 and 1572 to be completed and submitted to FDA to obtain their IND number. Reference to the appropriate DMF number under section 9 of form 1571 is in lieu of supplying the chemistry, pharmacology, and toxicology data required under section 12, subsection 7 and 8 on form 1571. Form 1572 is to be filled out by each subinvestigator involved in the study. Form 1572 is designed primarily as a statement of the qualifications of the investigators and it is not necessary to fill out section 8 if there is only one common protocol. Simply indicate "See Section 6a" on form 1571 on part 8. An information sheet containing further instructions on filling out these forms is also enclosed with the approval letter for human studies. If further clarification of the IND process is needed, the researcher may contact the FDA, Center for Drug Evaluation and Research, Rockville, MD at (202) 443-3510. The process is initiated by the researcher. FDA, upon receiving the information from the researcher, will assign an IND number and the application will be forwarded to the appropriate reviewing division. The reviewing division will send a letter to the investigator providing notification of the IND number assigned, date of receipt of the original application, address where future submissions to the IND should be sent, and the name and telephone number of the FDA person to whom questions about the application should be directed. Studies may not be initiated and shipments of test materials do not proceed until 30 days after the date of receipt of the IND by FDA. A study placed on clinical hold may not be conducted under the IND. The FDA will contact the investigator by telephone if the study is placed on clinical hold with a follow-up written explanation.

It is the responsibility of the researcher to notify the Charleston Laboratory, NMFS, if FDA places a clinical hold on the study. It is the investigators' responsibility to comply with applicable regulatory requirements and ethical standards of the FDA concerning the use of investigational drugs in humans. Once an IND number is issued by FDA, the researcher is instructed to send a copy to the NIH FOTMP office. A signed Waiver of Liability statement concerning the use of these materials must be signed and returned to the FOTMP office prior to shipment of the BTMs.

4) Non-U.S. Researchers

Researchers residing outside the U.S. are eligible to receive BTMs upon approval by the FOTMDC. Once approved by the FOTMDC, the Charleston Laboratory will send a letter stating that a number of conditions must be met by the researcher prior to BTM shipment (Appendix 2a and 2b). The Charleston Laboratory has formulated a Charleston Foreign Research Shipment (FRS) Committee to oversee compliance of foreign researchers with U.S. FDA regulations and to expedite shipment of the BTMs. Once these conditions are met by the researcher and verified by the FRS Committee, shipment of the BTM may proceed. The FDA has sent letters to the Charleston Laboratory authorizing shipment to researchers outside the U.S. when compliance with regulations have been completed.

5) Verification of Correct Mailing/Shipping Address

6) Technical Information

A cover letter, QA data sheet, instructions for storage, and a method for formulation of semi-purified animal diets with fish oils are included either prior to and/or with each shipment. The technical information referred to as a, b and c is provided for each researcher while d, e, and f are provided if pertinent to research protocol:

a. Complete Quality Assurance (QA) Data Sheet on each lot of BTM.

b. NOAA Technical Memorandum NMFS-SEFC-211; "Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil.[7]

c. 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 materials.

d. NOAA Technical Memorandum NMFS-SEFC-213; "Storage Stability of Steam-Deodorized Menhaden Oil in Soft Gelatin Capsules".[6]

e. NOAA Technical Memorandum NMFS-SEFC-222; "Evaluation of Flavors for Masking Sensory Attributes of Fish Oil".[5]

f. Technical information on the proper preparation and storage of animal diets containing highly unsaturated fatty acids (Appendix 4).

2.4 COORDINATION

As information is obtained on the type and quantity of BTM requested (either as a new request, continuing request or an ongoing study that needs additional BTM), the BTM is posted on the production schedule on the wall in the fish oil plant. At the same time production requests are also noted on written forms that are distributed to all project leaders and the program manager. The upper limit for lot size for bulk VDFO is 55 gal drum (195 kg). Efforts are made to consolidate requests and post the request as a full 55 gal. drum. Production is allotted a minimum of 5 working days to produce one 55 gal drum. The Production and Distribution Management Projects strive to coordinate the production of larger requests over a defined period of time in order to minimize Quality Assurance (QA) workload. Where requested quantities of VDFO warrant production of 55 gal, the VDFO are stored after processing in 55 gal food grade steel drums blanketed with nitrogen at 5°C. Quantities of VDFO less than 55 gal and specialty requests (eg. fish oil without antioxidants and fish oil with only TBHQ or tocopherols) are posted in the quantity requested, with some extra for archive and storage purposes, and stored in stainless steel vacuum vessels at 5°C. Requests for n-3 ethyl ester concentrate are requested in quantities of 5, 16, 35, or 55 gal. with an allowance of 5 days for production of 5 gal, 14 days for 16 gal, 28 days for 35 gal, 40 days for 55 gal quantity. The n-3 ethyl ester concentrate is stored in stainless steel vacuum vessels under nitrogen at -40°C until time for transfer to containers for shipment. Stainless steel pressure vessels are available in the following sizes for the storage of n-3 ethyl ester concentrate: 5, 16, 35 gal.

On the particular day when the production of the requested BTM is completed, Production assigns a 'lot' number to the BTM. Production will alert Distribution staff that a 'lot' is available and is currently being mixed. After adequate mixing, a sample is taken by distribution staff on the day the 'lot' is produced and submitted for QA analyses. If Distribution staff is not available the day a 'lot' is assigned, the production staff will take a sample, submit it for QA analyses, and place the 'lot' in storage. Samples are submitted according to procedures outlined by the QA project for sample submission.

* The following samples are taken for each 'lot' for QA analyses: 125 ml in a polyethylene container; 20 ml in a glass culture tube with a teflon-lined cap for PCB/pesticide analyses.
* The following amount of each 'lot' is archived at the same time that the lot is sampled for QA analyses: two 500 ml in nalgene polyethylene containers.
* The product remaining in the 20 ml glass tube after PCB/pesticide analyses will also be archived.
* All archive samples are placed in the freezer (-40°C) and logged in the Archive Record book.

A master chart serves to track a request from the time it is received through production, QA analysis, and shipment. A remaining balance is maintained on the initial requested amount for every 'lot number' and each type of BTM. Routine QA is conducted within a 3 week period for each lot of BTM. A QA report accompanies all 'lots' of BTMs to be used in human and animal studies. Signature by the QA project leader on the QA report for the BTM constitutes release of that particular 'lot' of BTM. An urgent request for BTM for ongoing animal studies may be met by working with the QA project for release of a particular 'lot' contingent upon specific analyses. A full QA report is provided to the researcher at a later date.

Coordination in the distribution project is facilitated by the use of several databases to record contacts with each researcher as well as with the NIH/FOTMP office. A database is maintained on the approved researchers detailing information on each researcher, each request, shipment and schedule of shipments for those doing long term studies. A database is maintained on the BTM inventory that accounts for each shipment of BTM. A FOTMAC Award Summary is updated on a monthly basis that describes each research request, status of the request and shipments made. In addition, to assist in the tracking of requests, the following files are maintained:

* U.S. requests - 1) approved (all requirements met), 2) approved (pending information on research request, waiting on IND approval, study start-up or continuing shipments) and 3) deferred (pending clarification, approval).
* Non-U.S. requests - 1) approved (pending information on research request or requirements for FDA export of test materials) and 2) deferred (pending clarification, approval).
* Requests not-approved
* Cancelled requests

3.0 DELIVERY FORM - BIOMEDICAL TEST MATERIALS

3.1 BULK

3.1.1 INTRODUCTION

The omega-3 fatty acids in the fish oil, the ethyl ester concentrates, and the ethyl esters of purified fatty acids (eg. EPA and DHA) consist of long carbon chains containing large numbers of double bonds. These double bonds are extremely susceptible to attack by atmospheric oxygen, and through this reaction, free radicals are formed, particularly hydroperoxides.

Oxidation of fats and oils proceed by a "chain" reaction which is initiated and propagated by formation of free radicals. Free radicals, once formed, cause a chain reaction which is self-perpetuating causing a type of degradation termed "autoxidation". Termination or inhibition occurs by removal or deactivation of free radicals prior to their catalyzing the autoxidation process. Antioxidants are often termed free-radical inhibitors since they function by interfering with the free-radical mechanism of autoxidation.[8] In autoxidation of n-3 fatty acids, the fatty acids are first converted to hydroperoxides. These are subsequently converted by further reactions to aldehydes, ketones, hydrocarbons and cyclic compounds. Aldehydes and ketones are the components which contribute "off-flavors" to foods, commonly recognized as rancidity. A final step in the autoxidation of n-3 fatty acids is polymerization of the material and the formation of polymers which may have health implications.

A key point in the chemistry of free radicals is that a minute quantity of the initiator (the initial free radicals produced by reaction with oxygen) can lead to high yields of oxidation products. Thus, it is important that the n-3 test materials are protected from exposure to air at all times and contain terminators (antioxidants) which will act to end the chain reaction. Precautions are taken during the processing of the BTM at the NMFS Charleston Laboratory to assure that the BTM are protected from oxygen and possible contamination. Small amounts of easily oxidized materials, known as antioxidants, are routinely added to the test materials. These materials act to scavenge free radicals by reacting with them more readily than do the n-3 fatty acids. The presence of antioxidants is thus intended to decrease the rate of autoxidation in the test materials. However, they can be added only in limited concentrations, and if the test materials are mishandled, even the antioxidant activity may be exceeded by the autoxidation process once it is initiated.

Alpha- and gamma-tocopherol and the antioxidant tertiary butylhydroquinone (TBHQ) are added to the fish oil and n-3 ethyl ester concentrate. The antioxidants interrupt the free-radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyl groups. Stable free radicals are formed that do not initiate nor propagate further oxidation of lipids. Tocopherols are natural antioxidants found in plant tissues existing as combinations of alpha, beta, gamma and delta homology. Antioxidant activity

decreases from delta to alpha while vitamin E activity increases in the reverse direction, with alpha-tocopherol the most abundant and active source of vitamin E. The tocopherols are highly miscible in fats and oils due to their extended alkyl chain. Tocopherols have GRAS status for use as food preservatives (21 CFR 182.3890) and are approved by the USDA for controlling oxidation in animal fats (9 CFR 318.7). Tertiary butylhydroquinone (TBHQ) is the chemical 2-(1,1-dimethyl ethyl)- 1,4-benzendiol (Chemical Abstracts Service Registry Number 1948-33-0). TBHQ has a melting point of 126°C to 128.5°C and is soluble in oil and fat and is considered the best antioxidant for protecting frying oils against oxidation.[9] The FDA permits TBHQ to be used to a maximum antioxidant content of 0.02% or 200 ppm, based on the fat content (CFR 172.185).

In order to enhance the use of BTM in clinical trials, it is important to produce a BTM that is indistinguishable from the placebo. The purpose of blind trials is to reduce the observer bias (subjective interpretation of results and heterosuggestion) and patient bias (subjective interpretation of symptoms and autosuggestion) by concealing the identity of the treatment administered either from the patient, the physician or from both, termed a double blind trial. In order to distinguish pharmacological effects from non-specific effects, a supposedly active drug may be compared to a placebo, pseudo-drug identical in appearance, but a pharmacologically inactive or innocuous substance. The first criteria in such a study is the need for an oral placebo which is identical in all respects to the active oral drug except that the active ingredient is missing. Particular features that require matching are the color, texture, taste, shape, size and mode of oral therapy. The use of fish oil or ethyl esters in human clinical trials requires the use of appropriate placebos. Most clinical studies thus far have used an oil such as corn oil, safflower oil or olive oil. While these oils are somewhat similar in texture, viscosity, and color, substantial differences exist in their sensory attributes of taste and odor. The use of fish oil and/or ethyl ester in single or double blind studies requires that the odor and flavor of fish oils be identical to the test treatment. In order to achieve this objective, a study[5] was initiated to develop a suitable placebo for use in double blind studies. The objective of this study was to determine the efficacy of flavors to mask the sensory attributes of fish oil so that adequate controls may be utilized for double blind studies. The studies demonstrated two possible alternatives for providing adequate controls for conducting double blind studies with fish oil. The research design may choose to flavor the fish oil very strongly with a peppermint or lemon-lime flavor or to flavor the control oil with a mild, fish oil flavor. Both approaches were shown to be effective in producing a fish oil treatment that was indistinguishable from the control oils of corn, olive and safflower.

3.1.2 PROCESSING

Distribution Management coordinates with Production on the processing schedule of BTMs for researcher requests. The bulk VDFO is placed in stainless steel vessels or food-grade 55 gallon steel drums under nitrogen and containing the antioxidants, alpha- and gamma-tocopherol and tert-butylhydroquinone (TBHQ). The drum contents are mixed thoroughly using a drum roller to ensure homogeneous dispersion of the antioxidant in the fin-

ished product. As soon as the oil is produced and mixed with the antioxidants, the Production Unit assigns a lot/batch number depending upon whether the oil will be used for researchers or for feedstock for production on n-3 ethyl ester concentrate and purified EPA and DHA. A sample of the 'lot/batch' is taken for QA/QC analyses and archive samples taken for each 'lot' for future reference. Antioxidants are added to the placebo oils prior to encapsulation and to placebo ethyl esters after the transesterification process which is described in the Production Manual[10]. Alpha- and gamma-tocopherol, and TBHQ are added to the vacuum deodorized fish oil at the Charleston Laboratory so that the final concentrations in the VDFO and n-3 ethyl ester concentrate are approximately 1.0 mg/g alpha-tocopherol, 1.0 mg/g gamma-tocopherol, and 0.02% Tenox 20A. Tenox 20A (Eastman Chemical Co., Rochester NY) is added at a concentration of 1 g/kg to provide TBHQ at a level of 0.02%, the FDA allowable limit for food products. Addition of Tenox 20A also adds the following components: 0.003% citric acid, 0.03% glycerol monoleate, 0.016% propylene glycol, and 0.03% corn oil. These antioxidants are added to increase the stability and shelf life of the test materials and allow the concentration of antioxidants in the placebo oil to be balanced with the fish oil. The objective is to attain approximately 1.0 mg/g for both alpha- and gamma-tocopherol. Since the VDFO is used as feedstock for the production of the n-3 ethyl ester concentrate and purified EPA and DHA, no further addition of antioxidants is required. The actual values on soft-gel capsules will vary depending upon a number of factors such as: experimental error associated with weighing and mixing the antioxidants, as well as analytical methodology. Antioxidants in the ester concentrate are those added in the initial vacuum-deodorized procedure: Vitamin E 5-67, Tenox GT-1, and Tenox 20A (all products are from Eastman Chemical Co., Rochester, NY). The antioxidants have been analyzed at the Charleston Laboratory and are tested for identity each time a new lot is received at the Charleston Laboratory, prior to addition to the processed oils. The antioxidants contain the following components:

Tenox 20A	Tertiary butylhydroquinone (TBHQ) 20% Citric acid 3% Glyceryl monoleate 32% Propylene glycol 15% Corn oil 30%
Tenox GT1	Mixed tocopherols (alpha, gamma, delta) 50% Edible soy 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)

Information is provided to the researcher on balancing antioxidant in control treatments (Appendix 1), including the offer of analytical support for determining the concentration of endogenous antioxidants in the control treatment being used. If antioxidant analyses are desired, the researcher is advised to send a 5 ml sample in an amber, glass or plastic container to the Charleston Laboratory. Analytical results of final concentration of antioxidants in the control treatment may also be verified upon submission of a sample after addition of antioxidants.

The bulk vacuum-deodorized fish oil (VDFO) is held in stainless steel tanks or 55 gal food-grade steel drums under nitrogen pressure. The VDFO is stored for a 6 month period at 5°C, while the n-3 ethyl ester concentrate test materials is stored for a maximum 3 month period at -40°C in which the products are used for research requests. At the end of the designated time periods, the BTM is disposed of or returned to production for reprocessing. The materials are warmed to ambient temperatures and transferred via nitrogen pressure to the specified size and quantity of nitrogen flushed containers for each researcher. Containers used for packaging the BTM are well-purged with nitrogen prior to filling with the BTM. The BTM is blanketed with nitrogen prior to capping the container. A Wheaton Perifill liquid dispenser is available for delivering small volumes of fish oils and n-3 esters at a delivery rate of 1.1 to 40.7 ml/sec depending on the tubing size and pump rate. The Perifill is a dual speed dispenser capable of dispensing 0.5 ml to 690 ml. The Perifill operates on a slow-quick-slow delivery rate sequence program. The slow start reduces initial splashing against a dry surface, then continues for the majority of the fill at a fast rate, and then finishes at a slow rate for the remainder of the cycle to prevent splashing, overflow or frothing. The dispenser is outfitted with silicone tubing and is compatible with both the fish oil and the ethyl ester. The Perifill dispenser can be contained in a glove box continuously flushed with nitrogen. The glove box is flushed with nitrogen prior to use and the empty containers flushed with nitrogen before placing them in the glove box for filling.

Purified fatty acid esters are obtained by supercritical CO_2 extraction and high pressure liquid chromatography. After processing the purified esters are transferred from the receiver of the product separator to a N_2-purged Nalgene amber, high-density polyethylene container, and stored at -40°C.

3.1.3 PACKAGING

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 is maintained if the material is bottled under nitrogen and stored at low temperatures. However, disadvantages of bottles include that they are not amenable to uses requiring controlled dose dispensing and require nitrogen purging between uses to maintain an inert atmosphere. Assessment of the research protocol assists in the determination of the best packaging regime that allows minimal opening of each container. The bulk BTM is custom packaged in a manner consistent with its use in the research protocol. 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 may conduct a simulated trial of the research protocol to obtain data on the stability and chemical characteristics of the BTM in order to address certain parameters and provide assistance.

The bulk oil is stored under appropriate conditions of temperature, humidity and light so that the identity, strength, quality, and purity of the BTMs are not affected. The containers used in packaging the test material must be compatible with the product it holds. There are a number of potential containers that may be utilized for packaging the test materials, including plastic and metal containers. Whether a metal can is made of aluminum or

steel or a combination of the two, the metal can is not inert with respect to its contents or its environment. This has led to the development of many different coatings to protect the container and contents. It is difficult to assure the completeness of the coating or to determine if defects are present inside the metal container. The favorable costs and the physical and chemical resistance characteristics are primary reasons that a high density polyethylene (HDPE) plastic container was chosen for use with the test materials. The containers were chosen to provide the most acceptable protection against external storage factors that may cause deterioration of the BTM, particularly light and oxygen. Polyolefins are high-molecular weight hydrocarbons which include low-density and high density polyethylene, polyallomer, polypropylene and polymethyl pentene. All are break-resistent, non-toxic, non-contaminating, and can withstand exposure to nearly all chemicals at room temperature for 24 hours. Polyethylene is formed by the polymerization of ethylene and classified according to the relative degree of branching (side-chain formation) in their molecular structure. High density polyethylene has minimal branching which makes it more rigid and less permeable than low density polyethylene. Polyethylene meets the requirement of the Food Additive Amendment of the Federal, Food, Drug, and Cosmetic Act. Polyethylene has been shown to be non-toxic to cells and distilled water often is stored in polyethylene containers. The HDPE plastic containers supplied by Nalgene Corporation (Rochester, NY) used for packaging and shipping of the bulk fish oil and n-3 ethyl ester concentrate are FDA approved for food use. These containers are utilized for shipment of all test materials that are used for human studies and the majority of animal experimentation. The Nalgene HDPE, amber bottles comply with the U.S. Pharmacopeia XXI requirements for maximum light transmission and are used to protect the test materials from light. The Nalgene HDPE, amber containers contain the following manufacturing specifications : maximum temperature 120° C, brittleness temperature -100° C, specific gravity 0.95, good resistance to oxygen permeability, excellent resistance to water vapor, and good chemical resistance for esters. The resin, Sclair 56B4, used in the Nalgene containers complies with the requirements of the Federal Food, Drug and Cosmetic Act and with Title 21 of the Code of Federal Regulations, Section 177.1520 (olefin polymers) and Section 178.2010 (antioxidants and stabilizers). As such, this resin may be used in contact with all types of food. The screw cap used for these containers is composed of polypropylene manufactured under Drug Master File #7325 with the FDA and conforms to FDA requirements under regulation 21 CFR 117.1520. The following specifications and stock numbers reflect the Nalgene, polyethylene food-grade containers used for packaging test materials for human and animal studies.

Container Specifications:

Amber plastic bottles	
Resin:	High density, linear polyethylene
Pigment:	Iron oxide
Density:	Polyethylene 0.95
	Polypropylene 0.90
Minimum Wall thickness:	0.020"
No mold release compound	

Nalgene Stock Numbers:

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

The containers used for shipping bulk oil are high density polyethylene that are consistent in size with the researcher's use of the BTM to avoid frequent warming, opening, and reclosing of the container of oil. The container size is stipulated based on information obtained from the researcher's experimental protocol with respect to diet preparation and/or BTM usage. In addition, a shipping schedule is arranged based upon the research protocol. The bulk VDFO, maintained at 5°C, and the n-3 ethyl ester concentrate, maintained at -40°C, are warmed to ambient temperature prior to transferring the test materials into containers. The transfer of BTM from storage vessels to containers for shipping is conducted the day of or the day prior to shipping the BTM. Larger size containers (>200 ml) 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 and fastened with tape to prevent accidental opening during shipping. The containers are placed in plastic bags and tied in case of spillage during shipping. Labels are made using computer fonts and a laser printer, photocopied onto self-adhesive label paper and applied to the bottles. The labels contain the following wording: "BIOMEDICAL TEST MATERIALS LOT XXXX Store at -40°C CAUTION: NEW DRUG LIMITED BY FEDERAL USE (USA) LAW TO INVESTIGATIONAL USE".

Good Manufacturing Practices are followed with some examples provided below:

* Initial testing demonstrated that the Nalge HDPE containers are not reactive with the product and does not alter the safety, identity, strength, quality and purity of the BTM. Stability testing indicated that the Nalge HDPE containers are compatible with the BTM; only these specific container types have been approved for use with requests for 2 L or less of BTM. All bulk materials for use in human studies are placed in Nalge HDPE containers. Requests for larger quantities for large animal studies are sent in 55 gal food-grade steel drums.

* Written procedures contained in an in-laboratory Standard Operating Procedures (SOP) of the Distribution Management Project provide details on the receipt, identification, storage, handling, sampling, testing and approval or rejection of containers.

* Containers are handled and stored in a manner to prevent contamination.

* Containers are stored off the floor and organized with suitable space to permit cleaning and inspection. Further, it is specified that no insecticides are sprayed in the packaging area.

* Requests for bulk BTM are custom-packaged by one person with verification of the type and quantity of BTM by a second person.

* All BTM 'lots' are archived in two 500 ml Nalge HDPE containers and stored at -40° C.

* A complaint file has been established for handling all complaints regarding the BTM. Written procedures describe the provisions made by QC of any complaint involving the BTM. A written record of each complaint is maintained in a file designated as such for a period of 3 years after distribution of the BTM as specified in the FDAs Current Drug GMP (1978 Final Rule). These records include the following information: product name and 'lot' number, name of complaintant, nature of complaint and the reply to complaint.

The containers are packed in corrugated boxes with appropriate packing material. Packing slips designating the contents of the box is sent with the shipment. BTM shipments in the U.S. are usually sent via Postal Express Mail overnight delivery service; large shipments are sent via refrigerated carriers. A record of all shipping information is included in a bound shipping log as well as into a computerized database. The distribution of each lot of test material can be readily determined. All shipments receive a cover letter describing the storage of the BTM, QA report, manual on analytical methods, information on the balancing of antioxidants in control oil (Appendix 1)and the formulation of semi-purified diets (Appendix 4). Shipments are made to researchers when the following requirements have been made:

U.S. Researcher

1) Approval letter for requested BTM
2) Signed waiver of liability
3) Human studies require IND; shipment may proceed 30 days after receipt of IND.

Non-U.S. Researcher

1) Approval letter for requested BTM
2) Signed waiver of liability
3) Letter from Ministry of Health with conditions stated in Appendix 2.

Bulk packaging of purified fatty acid ethyl esters (eg. EPA, DHA) are supplied to individuals conducting basic research into the mechanism of action of the specific polyunsaturated fatty acid. Gram quantities of purified test materials are dispensed in a N_2 glove box that has been well purged with N_2. The bulk fatty acid ethyl esters are transferred from bulk storage containers to brown glass or plastic vials sized for individual use of the contents, using disposable Pasteur pipettes. The vials used to package the purified EPA and DHA are amber in color and made of either HDPE (Nalgene, Rochester, NY) or glass (1.0 and 2.0 ml cryule vial; Wheaton, Millville, NJ) . The plastic containers are equipped with a amber, HDPE, screw-type closure while the cryule glass vials are sealed closed with a flame. The materials are packed in a

styrofoam container with dry ice for shipment to the investigator. The following sizes of Nalge containers are used for dispensing the purified esters:

Container Size	Stock Number
4 ml	2004-8125
8 ml	2004-9025
15 ml	2004-9050
30 ml	2004-0001
60 ml	2004-0002

3.3.4 STABILITY

Storage Recommendations

The NMFS Charleston Laboratory recommends storage conditions for the bulk fish oil and n-3 ethyl ester concentrate to the researchers based on QA/QC analyses of storage studies. Researchers are advised to store bulk fish oil and ester concentrates at 5°C if the entire contents will be used in two weeks or less. Any remaining oil should be well-blanketed with nitrogen prior to returning it to the refrigerator. If, however, the oil must be stored for more than two weeks, it should be frozen. The time required to thaw the oil will depend, of course, upon the size of the container. The container should be placed in a suitably sized container of water at about 50°C until the oil comes to ambient temperature. The contents of smaller containers can be mixed by gentle inversion of the container. Larger containers (5 gal and greater) can be mixed by bubbling **zero grade nitrogen** through glass tubing to the bottom of the container for 15-30 minutes. The esters should be stored at -40°C if possible, regardless of the rate at which they will be used. If only a portion of the n-3 ethyl ester is used, the remainder should be blanketed well with nitrogen and capped tightly before placing in the freezer. Stability studies are being conducted with the test materials in storage containers and formulated diets to provide reliable recommendations on storage parameters for maintaining the test materials at the production facility and providing information on stability and optimum storage conditions to the researcher.

Bulk Packaged Test Materials

Vacuum-Deodorized Fish Oil

A 12-month storage stability study has been completed[11] with the bulk vacuum deodorized fish oil (VDFO) containing tocopherols and TBHQ antioxidants stored at 5°C and -40°C (Appendix 3a). The stability study is being conducted using 100 ml aliquots in Nalgene (Rochester, NY) amber, HDPE containers stored at -40°C and 100 ml aliquots stored at 5°C in both Nalgene amber, HDPE and Alpha-Plastics (St. Louis, MO) amber, HDPE containers. Quarterly sampling of the bulk VDFO in 100 ml aliquots in Nalge HDPE amber containers stored at -40°C demonstrated very little change in the parameters measured; fatty acid

composition, iodine value, free fatty acid content, peroxide value, anisid ine value, antioxidant content and sensory attributes. The VDFO stored in Nalgene HDPE containers at 5° C had an increase in peroxide value (PV) from 0.4 meq/kg to 11.4 meq/kg in 6 months and 15.4 meq/kg in 12 months. The VDFO stored in Alpha-Plastic HDPE containers at 5° C had the highest PV at each time period increasing to 10.1 meq/kg at 6 months and 22.6 meq/kg at 12 months. All other parameters measured for the VDFO stored at 5° C had relatively little change over the 12 month period. One possible explanation for the differences in storage stability between the HDPE containers by the two manufacturers is that the Nalgene container had a superior bottle closure that provided an air tight closure. These analyses support the recommendation for storage of the VDFO bulk packaged in Nalgene HDPE at -40° C or less. Samples will continue to be analyzed quarterly over a period of two years to determine the stability of the VDFO.

Since bulk VDFO is provided to researchers occasionally without antioxidants to accommodate a specific research objective, s storage study was conducted to assess its stability. A 12-month storage study was conducted with 100ml aliquots of the same lot of bulk VDFO without antioxidants in HDPE amber containers at -40° C (125 ml, #2009-004; Nalgene, Rochester, NY), with amber, HDPE containers, (100 ml, #41872; 200 ml, #41876; Alpha-Plastics, St. Louis, MO.) and amber, glass containers (120 ml, #B7465-19; All-Pak, Pittsburgh, PA) at 5° C (Appendix 3b). At -40° C storage temperature, VDFO without antioxidants in Nalgene HDPE demonstrated no change in quality over a 12 month period similar to the VDFO with antioxidants (Appendix 3a) under similar conditions. Results indicate good stability when the VDFO was stored in the Nalgene HDPE containers at -40° C with little change occurring in the PV. The VDFO without antioxidants stored in the Alpha-Plastics HDPE containers had a high peroxide value of 22.6 meq/kg in 12 months storage time at 5° C. The peroxide value of the VDFO without antioxidants in the Nalgene HDPE containers were slightly lower than the Alpha-Plastics HDPE containers probably reflecting the oxygen permeability differences of the containers and the leakproof closure of the Nalgene container. Temperature appeared to have the most influence on stability of the test material in polyethylene containers. Storage of the VDFO at -40° C in HDPE containers provides adequate stability of the VDFO. The glass containers at 5° C provided stability similar to the VDFO stored in HDPE containers at -40° C. Glass containers provides good stability of the VDFO but have a greater potential for breakage during low temperature storage and shipping. Despite the difference detected in the PV during the 12 months of storage in different containers, no differences in product quality was detected in sensory product evaluations and only small decreases observed in the n-3 fatty acid content. In comparing the storage of the VDFO with and without the addition of antioxidants (alpha- and gamma-tocopherol, and TBHQ), container type and temperature appear to be more critical than antioxidant addition in protecting the BTM from oxidation with temperature having a predominant effect on the storage stability of the VDFO.

N-3 Ethyl Ester Concentrate

A 12-month storage study has been completed[11] for bulk n-3 ethyl ester concentrate stored in HDPE, amber containers at 5°C and -40°C (Appendix 3c). Results indicate good storage stability for the bulk n-3 ethyl ester concentrate stored in HDPE containers at -40° C for 9-12 months. Initial peroxide

value of of the n-3 ethyl ester concentrate increased from 5.8 meq/kg to 8.5 meq/kg at 3 months and did not change appreciably over the 12 month time period. The EPA and DHA content decreased, 3% and 5% respectively, to a greater extent than was found in the VDFO. There was no apparent change in the quality of the product as detected by sensory evaluation. It is recommended that bulk n-3 ethyl ester concentrate packaged in HDPE containers be stored at -40° C.

Most researchers conducting clinical trials select the soft gelatin capsules as the delivery form, but on occasion bulk n-3 ethyl ester concentrate may be selected as a dosage form. In order to be able to recommend reasonable storage conditions, a simulated trial was conducted in which bulk n-3 ethyl ester concentrate was stored at 5° C in amber, nalge HDPE and opened three times daily. The peroxide value at Day 1 was 2 meq/kg and increased to 21 meq/kg on Day 7. Caution is recommended in the use of bulk n-3 ethyl ester concentrate for clinical trials and packaging of daily doses would be optimal in terms of stability.

Purified EPA and DHA

Purified EPA and DHA test materials are available in bulk form for research studies. The following two objectives are being addressed in a stability study with the purified fatty acid ethyl esters: 1) define and recommend storage conditions for the purified test materials to the researcher and, 2) determine the conditions and length of time that the purified fatty acid ethyl esters can be stored after processing. A storage stability study currently is examining the role of temperature, oxygen and container type on the stability of purified fatty acid ethyl esters of EPA and a study with DHA will be performed also. EPA was transferred into two different types of containers: 1) glass cryule vials (1 ml, #651486; 2 ml, #651483; Wheaton, Millville, NJ) at -40° C and 2) HDPE vials (#2004-8125; Nalgene, Rochester, NY) at -40° C and -90° C. The vials were flushed with nitrogen and the plastic vials capped while the glass vials were covered with parafilm and placed in a glovebox. Once inside the glovebox, the EPA was transferred into the vials with a glass syringe and the plastic vials capped with screw tops and the glass vials resealed with parafilm. The samples in glass vials were removed from the glove box, the parafilm removed, and placed on a manifold, evacuated, and flushed with nitrogen 10 times. The vials were sealed with a flame and placed in appropriate storage conditions. In addition to EPA transferred under glovebox conditions using a nitrogen atmosphere, samples were also aliquoted into containers under normal laboratory benchtop conditions to determine the effect of oxygen on stability. The samples will be subjected to the following analyses over a 12 month period: fatty acid profile, tocopherols, peroxide value, sensory attributes, and moisture. Preliminary results for the 3 and 6 month sample periods indicate good storage of the EPA under both conditions for all parameters measured. The PV results for the glass and plastic storage containers at 3 months were 1.3 meq/kg and 1.8 meq/kg, respectfully. After 6 months, the PV value of the EPA in the glass container was 1.3 meq/kg and 2.6 meq/kg in the plastic container. The PV results also indicate little difference between packaging within a nitrogen flushed glove box and packaging with nitrogen blanketing.

Test Materials in Formulated Diets

In addition to conducting stability and container compatibility studies of the bulk test materials, the BTM Program has participated in conducting stability studies of the bulk test materials in formulated diets. The FOTMAC committee recommends to the researchers precautions to be taken, when formulating experimental semipurified diets (Appendix 4). It is recommended that experimental diets be prepared twice weekly, stored in air-tight, non-plastic containers at -70°C if possible, in the dark. Animals should receive fresh food on a daily basis with removal of any residue before addition of fresh food. Each researcher is advised to quality assure and validate their research protocol to determine stability of the formulated diet and whether the diets need to be prepared on a daily, weekly or biweekly basis. A collaborative study with Dr. Karmali of Rutgers University is currently being conducted on the stability of the vacuum-deodorized fish oil and a control vegetable oil as well as the n-3 ethyl ester concentrate and a control vegetable oil ethyl ester in formulated diets. Preliminary results indicate that the fish oil and placebo oils (corn oil, olive oil ethyl ester) remain stable under storage conditions at -20°C and 5°C mimimally for 28 days. While the olive oil was stable at 23°C for 56 days, the PV of the corn oil increased to 10.2 meq/kg, and the fish oil increased to 25.6 meq/kg. The n-3 ethyl ester concentrate had much higher PV of 12.3 meq/kg after processing into formulated diets and increasing to 25.8 meq/kg, 73.7 meq/kg, and 246.8 meq/kg, after storage for 28 days at -20°C, 5°C, and 23°C, respectively. After 56 days, the n-3 ethyl ester concentrate increased to 58.0 meq/kg, 139.0 meq/kg, and 638.5 meq/kg at -20°C, 5°C, and 23°C, respectively.

Test Materials in Storage Containers

Fish oil stored in 55 gal steel drums has excellent stability over a 9 month period. Peroxide values after 4 months of storage increased from 0.38 meq/kg to 0.56 meq/kg. Fish oil ethyl ester concentrate stored in stainless steel pressure vessels under nitrogen pressure is less stable and has a increase in peroxide value from approximately 4-5 meq/kg to 9-10 meq/kg after 3 months.

3.2 SOFT GELATIN CAPSULES

3.2.1 INTRODUCTION

There are relatively few manufacturers of soft gelatin capsules primarily due to economic, patent, and technological factors. The soft gelatin capsules are used in a wide variety of industries because of their special properties, but they are used most widely in the pharmaceutical industry to provide a solid dosage form containing liquid medication in an accurate and uniform dosage.[7] Soft gelatin encapsulation of fish oils and n-3 ester concentrates provides a convenient measured dose delivery having good stability. Since the gelatin shell is an effective oxygen barrier, there is reduced concern relative to ingredient stability. Most stability problems are associated with the available moisture from the gelatin shell which, when absorbed into the capsule content may cause areas of high concentration of water-soluble solids, leading to ionization and interaction of the solids[12].

Soft gelatin capsules are composed of water, gelatin and a plasticizer, usually glycerin or sorbital. The encapsulation can be performed using water-imiscible liquids, water-miscible liquids containing <5% water and dry powders. Temperature and humidity effects on gelatin capsules are well documented. Transient effects, such as brittleness, can be caused by low humidities (<20% R.H.), and either low temperatures (<2° C) or high temperatures (>37° C) or combinations of these conditions. The capsules will regain moisture in proportion to its gelatin and glycerin content as conditions are restored. High humidities (>60% R.H.) at 21-20° C can produce non-reversible changes in the unprotected capsule resulting in a capsule that is soft and tacky. As conditions return to moderate conditions, the capsules may become cloudy and stick together.

3.2.2 PROCESS

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 within 1 to 3 percent. Capsules are manufactured and partially dried in the following three continuous steps:[12]

1) Two gelatin ribbons are prepared, automatically and continuously, and fed with the product to the encapsulating mechanism.
2) 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.
3) The formed capsules are automatically conveyed to and through a solvent wash and initially 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 the solvent, they are placed in a forced air drying tunnel for 1 hour at 80°F. This drying process removes 4-5 percent 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 at equilibrium.

3.1.5 PACKAGING

The test materials were encapsulated by a commercial encapsulator 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).

The encapsulation process employed during encapsulation of the test materials was modified to incorporate the following changes in the standard encapsulation operating procedures: 1) the addition of specified amounts of antioxidants in the test materials and placebo oils (see Sec. 3.1.2); 2) protection of the test materials from exposure to air prior to encapsulation; 3) protection of the encapsulated product during packaging by nitrogen blanketing each bottle of capsules prior to application of the heat seal, and 4) brown, polyethylene bottles were used to provide the best protection for the test materials while facilitating shipping of the product.

Fish oil and placebo soft gelatin capsules were produced with a fill weight of 1000 mg/capsule (#20 oblong) and bottled with 100 capsules/bottle. Products produced by contract with the Chase Chemical Co., Newark, NJ, include: 500K soft-gel capsules of steam-deodorized fish oil, and 250K each placebos of olive, corn and safflower oils. These soft gelatin capsules were produced using titanium dioxide, an opacifying agent, in the gel formulation. To each kilo of steam-deodorized fish oil, 1 gm of Eastman Tenox 20A (20% TBHQ, 3% citric acid, 30% corn oil, 32% glycerol monooleate, 15% propylene glycol) and 1 gm of alpha-tocopherol were added prior to encapsulation. Although no antioxidants were added to the placebo oils, they all contain natural levels of tocopherols. Nitrogen was used during the preparation of the oils but not the packaging process. These capsules were packaged 100 capsules per amber glass bottle (each bottle with an internal plastic seal under the screw cap lid), 24 bottles per shelf-pack, 3 shelf-packs per master case (72 bottles/case). These capsules are being stored at 5°C.

Clear soft gelatin capsules of steam-deodorized fish oil (175K) and capsules of corn oil (164K) were produced and packaged in amber glass bottles (100 capsules/bottle) by General Nutrition Products (GNP), Greenville, SC. The antioxidants (alpha-, gamma-tocopherol, and TBHQ) were balanced in the fish oil and corn oil prior to encapsulation so that the final concentrations were: 1.3 mg/g alpha-tocopherol, 1.2 mg/g gamma-tocopherol, 0.02% TBHQ. Addition of TBHQ also added the following components: 0.003% citric acid, 0.03% glycerol monooleate, 0.016% propylene glycol; and 0.03% corn oil. These capsules 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. Tamper-evident seals were applied to the outside of the bottle cap. The fish oils and placebos were transported to the Charleston Laboratory and are currently being stored at 5°C. Additional quantities of BTM encapsulated by GNP include the following amounts of soft gelatin capsules: 326K fish oil capsules, 1,056K n-3 ethyl ester concentrate, 71K corn oil, 76K olive oil, 54K safflower oil, 16K olive oil ethyl ester and 14K corn oil ethyl ester. GNP has been awarded a long term contract for the encapsulation of the BTM. A NMFS BTM Program representative inspected and provided oversight at both encapsulation facilities during encapsulation of the fish oil and placebo oil(s). It appeared that good manufacturing practices were being utilized at both facilities.

Several modifications of the encapsulation process at GNP were incorporated during encapsulation of the BTM to minimize exposure of the BTM to oxygen and the maintain the high quality of the BTM. These modifications include:

* Initial transfer of the 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 was altered so that the contents were transferred by a transfer line using nitrogen pressure into a container that had been flushed with nitrogen. The container was then blanketed with nitrogen and closed with an air-tight cover during mixing and prior to encapsulation.

* During the packaging of the products, nitrogen flushing of the bottles containing the capsules was incorporated at the bottle slow down point, allowing a 3 sec flush, prior to application of the heat seal.

* Amber, polyethylene bottles were used to provide the best protection for the test materials while facilitating shipment of the test materials.

The components used by GNP in formulating the capsules material are gelatin (Appendix 5a), water, and glycerin (Appendix 5b). Perchloroethylene is used as a washing solvent by GNP during the manufacturing of soft gelatin capsules of n-3 ethyl ester concentrate. The following information was obtained from GNP regarding the use of the solvent in the manufacturing of the soft gelatin capsules:

1. The solvent in use (percholoroethylene) meets USP specification requirements for internal use in humans.

2. The test method consisted of headspace analysis/gas chromatographic anlayses for 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 method, the capsules contain no detectable level of percholoroethylene residue: the value fell at or below the detection limit, of 0.4 - 0.5 ppm. 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 capsules of the encapsulated oils for coliforms, E. coli, and Salmonella using the procedures presented in the QA Methods Manual[5].

The amber plastic bottles used for the capsules of BTM meet USP XXI container specification (Appendix 5).

Product identification and information contained on pre-printed adhesive labels are applied on-line. All labels contain 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". Tamper-evident closures and/or an inner heat seal were applied to all bottles encapsulated by GNP. The caps were secured with breakable bands around the bottle top that separate when the cap is opened. The capsules are currently being stored at 5° C.

3.1.4 STABILITY

The nature of the materials comprising the capsule shell indicates that the unprotected soft gelatin capsules (i.e., capsules that can breathe) rapidly reach equilibrium with the atmospheric conditions under which they are stored. The effects of temperature and humidity on these gelatin capsules requires proper storage and packaging conditions.

The physical stability of soft gelatin capsules is associated primarily with the gain or loss of water by the capsule shell. If this is prevented by proper packaging, the capsule will have satisfactory physical stability at temperatures ranging from above freezing to over 120°F. However, as humidity increases, the shell of the unprotected capsule picks up moisture in proportion to its glycerin and gelatin content. The total moisture content of the capsule shell, at equilibrium with any given relative humidity within a reasonable temperature range, approximates the sum of the glycerin and gelatin content when held separately at the stated conditions. Low humidity (< 20% R.H.) coupled with either low temperatures (<35°F) or high temperatures (>100°F) produce only transient effects on the capsule. The capsule will return to normal when optimum storage conditions are restored. High humidities (>60% at 70°F to 75°F) produce lasting effects on the capsule shell, since as moisture is absorbed, the capsules become softer, tackier, and bloated. Capsules containing water-soluble or miscible liquid bases may be affected to a greater extent than oil-based capsules, owing to the residual moisture in the capsule content and the dynamic relationship between the capsule shell and the capsule fill.

A 12-month storage study has been completed on the encapsulated steam-deodorized menhaden oil capsules stored in glass, amber bottles at 5°C. Analytical evaluation of the BTM in soft gelatin capsules found that there was little or no change in the composition or quality of the encapsulated oil over the 52 week storage study[6]. The concentration of EPA, DHA and total n-3 fatty acids, antioxidant concentration, moisture, flavor and odor characteristics were measured on capsules stored at 5° C and ambient humidity, the same conditions as those used for storage of all BTM capsules. The study design consisted of randomly selecting one bottle every four weeks for a series of assays to determine product stability. Continued analyses of these capsules, performed after the publication of the 12-month study, showed no change in oil quality after 24 months. A storage stability of vacuum-deodorized fish oil

(VDFO) in soft gelatin capsules will therefore encompass analyses only at 1, 12 and 24 month time points. The data obtained thus far for the VDFO is summarized in Appendix 3d.

A 12-month storage study has been completed on the encapsulated N-3 ethyl ester concentrate capsules stored in amber, polyethylene bottles at 5°C. Analytical evaluation of the encapsulated n-3 ethyl ester concentrate (Appendix 3e) demonstrated relatively little change in the PV although a 2% and 3% loss was observed in EPA and DHA content, respectively, and decreases in the alpha- and gamma-tocopherol content.

In addition, a 15 day simulated trial of opening and closing a bottle of n-3 ethyl ester concentrate three times a day at room temperature versus refrigerated temperature revealed relatively little difference in compositional changes between the two temperatures. The capsules maintained at refrigerated temperature reflected a change in PV from 1.95 meq/kg to 2.76 meq/kg while the capsules held at room temperature had a change in PV from 1.95 to 2.57. This trial was performed in order to recommend storage conditions of capsular test material upon opening. The data indicate that clinicans may recommend that patients store the capsules at room temperature after opening.

3.3 AEROSOLS

3.3.1 INTRODUCTION

One of the objectives of the Biomedical Test Materials Distribution Management Project is to investigate feasible dosage delivery systems that maintain the high quality of the test materials and facilitate research protocols. The objective of investigating an aerosol delivery form of the BTM is to evaluate aerosol containment in maintaining the initial high quality of the test materials, particularly the highly oxidative ester concentrates, and also to enhance the flexibility and ease of test material use. Standard aerosol products consist of a liquid product or concentrate mixed with a propellant under pressure and contained in glass, plastic or metal containers, that has been sealed by crimping to a dispensing valve. The product is protected from oxidation since it can use an inert propellant such as nitrogen. Pressurized cans with metering valves may be a useful and convenient method for the storing and dispensing of omega-3 materials. The BTMs are currently available in bottles and gelatin capsule delivery forms. While these traditional delivery modes have their advantages, they also have some disadvantages. Bottles are normally sealed under nitrogen and provide good stability of the BTM, but after opening, the contents are exposed to oxygen. Gelatin capsules may be susceptible to damage in humid, cold or warm air, they do have size limitations, and some individuals experience difficulty in swallowing several capsules at a time. Current interest in the BTMs for clinical trials warrants the investigation of pressurized containers for storing and dispensing BTMs and the nature of the test materials mandate testing to confirm the stability of the BTM and the packaging components in the aerosol system.

3.3.2 PROCESSING

In order to assess the use of an aerosol delivery system for the BTM a small scale laboratory for aerosol experimentation was equipped with a can crimper-3000-C ADJ-KM, gasser-shaker unit and accessories for packaging from Aerosol Laboratory Equipment Corp., Walton, NY. The crimper was modified by BLM Packaging Inc., Greenwich, CT for use with the Lablabo metered valve. Lablabo valves for metering heads, 1" spouts, 21mm length, 1 ml metering heads, shields, caps, pressure gauge were used. A glove box was used for filling and crimping aerosol cans within a nitrogen atmosphere.

The following studies were initiated to investigate the feasibility of aerosol storage for the BTM: 1) a valve compatibility study and 2) a 30 day accelerated storage study. A valve component compatibility study was begun to investigate whether the valve components in the metered dispensing valve would pose any problems with the BTMs. The eight valve components (valve body, butyl cup gasket, cup gasket, internal gasket, housing, o-ring, spring, and stem) were individually placed in glass containers (3 replicates per component) with teflon-lined caps in either VDFO or ethyl esters for a period of 6 months. These components are being visually observed for a period of 6 months for changes in appearance (shape, texture, etc.)

After 6 months, samples of the BTMs will be subjected to gas chromatography headspace analyses to determine if migration of valve components occurred. A 30-day accelerated storage aerosol study was started using VDFO and ethyl ester. These BTMs were filled in six monobloc cans supplied by Boxal, Inc., Cranbury, NJ with 2 different linings (FDA approved epoxyphenolic and FDA approval pending Microflex, a vinyl organosol). Aerosol containers were flushed with nitrogen, sealed with a layer of plastic and placed inside a glovebox. Once inside the glovebox, the containers were filled with the BTM and the valve crimped in place. The lablabo metered valve was used without the spring component since it was not a required component and may result in rust formation. The filling specifications were as follows: product volume of 80 ml, can volume of 145 ml and 70 psi of nitrogen pressure. Crimping dimensions were as follows: diameter of 27.20 mm and a height of 5.35 mm. The 12 cans were placed in an oven at 140°C for a 1 month period. At the end of that time period, the oil was expelled through the metered valve and submitted for QC analyses. After the oil was expelled, the pressure was released and the can was cut in half for visual inspection. While several of the cans lost pressure, visual inspection revealed no defects in the lining of the can or valve components. A complete side cut revealed no traces of damage to the varnish lining of either can. The study of components also found no swelling on any of the cans nor traces of corrosion. Leakage around the valve stem was not evident, an indication of product compatibility with the gasket materials. A study of the functioning of the aerosol containers revealed good precision and repeatability in the dosage delivery. Longer term stability studies should be performed to assess the performance of the valve and the can linings with the VDFO and n-3 ethyl ester concentrate.

3.3.3 PACKAGING

Since the metering chamber of the Lablabo valve is located outside of the container, the metered valve offers the following advantages: 1) product flow is not dependent upon pressure variations of the propellant which means the dose is always constant and precise, 2)product fills the metering chamber of which the volume is predetermined and expelled through an elastic membrane and 3) foreign particles are prevented from entering the filled product.

The basic components or machines required to package aerosol products are more unique and complicated than other rigid containers filled with liquid. The selection of machinery for aerosol packaging is primarily determined by the production speed desired, price, type of product, container and propellants that are to be utilized.

3.3.4 Stability

While an in-house small scale laboratory for aerosol containment was designed for testing the feasibility of an aerosol delivery system, a commercial source was also used to evaluate the stability of the BTM in aerosol form. The VDFO and n-3 ethyl ester concentrate were packaged with nitrogen as the propellant in pressurized containers by Applied Laboratories, Columbus, OH. Duplicate cans were stored at room temperature and submitted for analyses of PV, fatty acid composition, and sensory analyses at 0, 6, and 12 months.

Prior to taking a sample for analyses, residual material was expelled from the delivery tip. Results indiicate that the initial PV of 0.9 meq/kg of the VDFO increased to 4.3 meq/kg immediately after processing and then to 9.3 meq/kg in 6 months. The PV of the n-3 ethyl ester concentrate increased to 7.19 meq/kg immediately after processing and the contents could not be expelled for the 6 month time point. The stability studies conducted with the test materials packaged by a commercial aerosol filler indicates product degradation over the 6 month time period as assessed by an increase in PV followed by a decreased PV. The aerosol containment of the test materials offered no increase in protection for the bulk test material than packaging in HDPE containers at 5° C. Packaging the test materials in HDPE containers and storage at -40° C provided superior stability when compared to the aerosol delivery form. Further, valve clogging problems developed in the aerosols containing the n-3 ethyl ester concentrate that were sampled over the 6 month period in which a new can of product was needed to obtain a sample. However, it should be noted that the stability of the test materials would be expected to improve if the commercial aerosol filler used evacuation techniques that would ensure removal of all oxygen.

An omega-whip dosage delivery system, marketed by Product Resources International (PRI), NY, for pharmaceuticals and nutritionals may have potential application as a dosage form for fish oil, an alternative to soft-gelatin capsules. Link laboratories developed the edible whip technology, a simple aerosol foam that makes possible the delivery of up to 50% solids without valve clogging. The omega-whip dosage form is an aerosol-whipped product that is dispensed into a spoon. The company has developed an "Omega Whip" consisting of a Japanese sardine oil. One teaspoon of the whip equals two and one-half one gram capsules of fish oil. The advantage claimed for this form is the ability to provide a large dose (2.5 gm per teaspoon) of omega-3 fish oils in a palatable, easy-to-take form, thus increasing patient compliance. The use of this form may be advantageous in clinical studies by providing a placebo oil that is similar in taste and odor to the fish oil. It consists of a basic formulation of 5% granular lecithin as a foaming agent, 90% soybean oil and 5% propellant (food grade propane). The proportion of the oil is adjusted when the active ingredient is added, or in the case of fish oil the oil is the active ingredient. All of the excipient ingredients are approved by the FDA for use in food products. Link Laboratory has found that microorganisms cannot grow in this system since the saturated sugar solution and the closed contained system prevents the growth of microorganisms. Even the spout, where the product is exposed to air, is free of microbiological contamination according to PRI. The use of an edible whip does an excellent job in masking the taste of fish oil and has a high potential for consumer acceptability and compliance. Since the formulations are anhydrous, aluminum and steel cans may be used. The system is said to be chemically inert and will not promote corrosion. In order to explore the potential use of the edible whip, the VDFO, n-3 ethyl ester concentrate and corn oil were formulated an edible whip by Product Resources, Int., NY. The following list comprise the ingredients in the BTMs formulated as an edible whip: BTM (fish oil, n-3 ethyl ester concentrate, or placebo), ascorbic palmitate, ascorbic acid ultrafine, TBHQ, sugar (12X), aspartame, colloidal silicon dioxide, diacylycerol tetraoleate, sorbitan monostearate, vitamin E, FM lemon mint, FM bavarian creme, and nitrogen as the propellant. Limited studies on the stability of the BTM formulated as an edible whip are being conducted. Analy-

ses of the VDFO, n-3 ethyl ester concentrate and corn oil formulated as an edible whip and stored at room temperature have been tested at 0, 3, 6, 9, and 12 months after processing (Table 1). Analyses of all three BTM show a marked increase in PV immediately after processing. Only a relatively small increase in the VDFO occurred after the initial processing, while the corn oil continued to increase. The n-3 ethyl ester concentrate at 6 months exhibited a decline in PV. One possibility for the decline may be the formation of polymers by the cross-linkage of peroxidized fatty acids.

TABLE 1. EDIBLE WHIP FORMULATION OF VACUUM-DEODORIZED FISH OIL (VDFO), CORN OIL AND N-3 ETHYL ESTER CONCENTRATE (N-3 CONC) STORED AT ROOM TEMPERATURE IN AEROSOL CANS.

TIME	PRODUCT	PV[1]	EPA[2]	DHA[3]	TOTAL[4] N-3	TIO[5]	TIF[6]
INITIAL	CORN	2.4	<0.05	<0.05	8.8	2.8	2.3
	VDFO	1.2	136	81	308	3.8	4.2
	N-3 CONC	1.9	414	236	788	4.9	5.3
0	CORN	4.1	<0.05	<0.05	6.1	4.2	4.9
	VDFO	9.2	106	64	230	5.5	6.2
	N-3 CONC	12.5	335	187	622	4.4	6.4
3	CORN	9.3					
	VDFO	12.1					
	N-3 CONC	12.2					
6	CORN	14.0	<0.05	<0.05	5.8	3.4	5.2
	VDFO	9.5	94	56	205	3.8	5.7
	N-3 CONC	2.9	284	160	528	3.9	7.8
9	CORN						
	VDFO						
	N-3 CONC						

1 PV - peroxide value (meq/kg)
2 EPA - eicosapentaenoic acid (mg/g)
3 DHA - docosahexaenoic acid (mg/g)
4 Total N-3 - total n-3 fatty acids (mg/g)
5 TIO - total intensity of odor (based on rating scale 0-15, 15 maximum intensity)
6 TIF -total intensity of flavor (based on rating scale 0-15, 15 maximum intensity)

Several containers have been sent to researchers involved with clinical trials to assess the possible interest and potential of using the edible whip formulation with the BTMs in human clinical trials. A summary of opinions rendered by four clinicans evaluating the edible whip were:

Overall rating of product:	good-3, very good-1;
Advantage over soft-gelatin capsules:	yes-3, no-1
Advantages:	fewer # doses/day tasty placebos can be made, better taste, no burping fish taste
Disadvantages:	unable to assess compliance by pill count; difficult to determine dose; concern over oxidation, aftertaste
Increased patient compliance:	yes-3, no-1
Interested in using product:	yes-3, no-1
Suggestions:	try to remove aftertaste, list suggested uses and inappropriate uses

3.4 MICROCAPSULES

3.4.1 INTRODUCTION

The rapid expansion of research on the health consequences of the n-3 polyunsaturated fatty acids (PUFA) has shown a potential beneficial role in preventing and/or ameliorating a number of diseases. Determining the scope of this potential as well as any accompanying adverse consequences will require additional clinical, biochemical, and physiological studies with higher purity test materials. The use of higher purity test materials, n-3 fatty acid ester concentrates (85% purity) and individual fatty acids (94-99% pure), however, introduces to the research community a new set of experimental conditions and handling problems owing to their markedly increased oxidative reactivity. The propensity of the PUFA to oxidize is exacerbated by processing into delivery forms, whereby natural levels of antioxidants are decreased. Improper handling may lead to oxidation of the PUFA and the use of oxidized PUFA can produce very misleading results. It is essential that oxygen be kept from the test materials. In the manufacturing process, nitrogen or argon gases can be used to limit exposure to oxygen. However, the use of the materials in humans and animals requires other consideration. In human experimentation, the PUFA can be packaged in a number of ways, such as soft gelatin capsules, so that they do not come in contact with oxygen prior to ingestion. In contrast, limiting oxygen exposure in animal diets is not readily achievable. In those experimental models where the material is to be fed as part of the diet, it is particularly difficult to control oxidation. Although oxidation can be controlled through the use of antioxidants, the relative efficacy of antioxidants with different test materials is not easily determined. Another approach used is to mix the test oils with the basal diet each morning and discard the uneaten portion at the end of the day. All dietary materials are kept in frozen storage until use and the test oil material is maintained in an inert atmosphere. However, the test material is still exposed to oxygen from 8 to 12 hours. In addition, the diets must contain antioxidants precluding the feeding of an antioxidant-free treatment.

Microencapsulation is a mode of delivery that may inhibit or eliminate the problems associated with oxidation of fish oils and n-3 ester concentrates during diet preparation. Microencapsulation has flexibility with potential application for use in animal feeding studies as well as facilitating human use of refined fish oil and concentrated n-3 PUFAs. Currently, many drugs, diagnostic agents, and other health care-related products are microencapsulated by pharmaceutical and medical companies. Microencapsulation is common to other industries such as food, cosmetic, horticultural, paint, photographic, computer, fertilizer, adhesives, cleaning and aerospace industries. Microencapsulation is now the most frequently employed method of producing controlled release dosage forms[13]. There are many reasons why drugs and related chemicals are microencapsulated. Microencapsulation has been employed to provide protection to the core material against atmospheric effects, as an aid in handling and storage, to disguise unpleasant taste and odor, to reduce gastric irritation, and to provide sustained release. Microencapsulation of fish oils and n-3 concentrates could improve the stability of dose formulation, prevent loss due to volatility, overcome palatability problems, and decrease animal stress via force-feeding thereby eliminating

daily animal handling and minimizing hazards to personnel. Microencapsulated chemicals offer significant safety and economic advantages for dose administration of reactive and/or volatile test chemicals. Microencapsulation of test materials allows the release of the product when the test animal chews the capsule or when the capsule dissolves in the animals digestive tract. With microencapsulation the use of fish oils and n-3 esters or triglyceride concentrates without antioxidants may be permitted. This would be desirable in testing the fish oil or n-3 concentrate in experimental situations in the absence of any effects derived from antioxidants.

However, caution must be exercised as several issues need to be addressed prior to promoting the use of microencapsulated fish oil and/or n-3 concentrates. The major concerns involving the microencapsulation of fish oils are centered on the questions of stability, acceptability, and assimilation of the product. Studies need to be performed that address questions pertaining to the shelf life of microencapsulated products to determine how long the preparation can be stored in a particular carrier before it degrades. Studies should be performed that 1) ascertain the quality of the product immediately after microencapsulation and assess the relative stability and 2) correlate the product stability with animal feeding trials to determine the acceptability and assimilation of the microencapsulated test material. An interagency subcommittee (FDA, USDA, NIH, ADAMHA, DOC) was formed to initiate an investigation of microencapsulating fish oils and concentrates of n-3 concentrated PUFA as feasible dosage forms[14].

3.4.2 PROCESSING

The biomedical test materials, vacuum deodorized fish oil (VDFO) and n-3 polyunsaturated fatty acid ester concentrate produced from menhaden fish oil were microencapsulated by two different laboratories, Laboratory A and Laboratory B. Laboratory A produced microcapsules utilizing a solidified emulsion patent pending process. When possible during production the test materials were maintained under a blanket of argon to minimize oxidation. Laboratory A formulated the matrix for all test materials using the same lot of 100% starch. The process basically involves the formation of an emulsion formulated with food grade ingredients which is sprayed through a nozzle, solidified by temperature and evaporative processes, and emerges as minute stable particles. The microcapsules were prepared under controlled temperature to allow approximately 40% of the weight to be the desired product. Sizing was conducted to produce a particle size range of 100 um to 400 um. The encapsulation process was performed at a temperature of 60°C and the material to be encapsulated was held in an argon atmosphere. The capsules were formed instantaneously and excess moisture was evaporated in an inhalation chamber during a 2-3 day period with equilibration at a constant temperature (25-30°C) and 45-50% humidity. Equipment was thoroughly cleaned between each run. Standard procedures include wipe tests and analyses for prior materials that were encapsulated to ensure no contamination. Laboratory B also employed a patent-pending process in the commercial production of a microencapsulated fish oil. The test materials were held under nitrogen prior to production of the microcapsules. The shell matrix used by laboratory B is comprised of 48% gelatin and 2% cellulose.

The antioxidants, alpha- and gamma-tocopherol and tertiary butylhydroquinone (TBHQ), were added in a comparable manner to the fish oil, and n-3 ethyl esters. The antioxidants were thoroughly mixed with the product in an inert atmosphere. A control substance encapsulated by both laboratories consisted of corn oil containing the same concentration of antioxidants (alpha- and gamma-tocopherol; TBHQ) and the same matrix as for the fish oil and ester.

3.4.3 Packaging

Biomedical test materials were microencapsulated by contractual arrangements for determining the feasibility of microcapsules as a dosage form. The materials were microencapsulated by the two different laboratories within 10 days of each other. After microencapsulation, the material was shippped to the Charleston Laboratory and aliquoted into various size containers within a nitrogen-flushed glove box for conducting stability and animal feeding studies. The microencapsulated fish oil/ester/corn oil were packaged in amber, polyethylene containers and flushed with nitrogen prior to sealing. These microencapsulated materials were subjected to quality assurance and quality control analyses at the NMFS Charleston Laboratory. The microencapsulated products were placed at three temperatures (-20°C, 5°C, and 25°C) for conducting storage stability studies. A monthly sampling scheme for QA/QC analyses was initiated for the material held at 5°C, while the material stored at 25°C and -20°C was subjected to analyses at 6 and 12 months. The animal feeding studies were conducted with microencapsulated material stored at 5°C.

3.4.4 STABILITY

The microencapsulation feasibility study[14] provided results that will enable NMFS-Charleston Laboratory Biomedical Test Program to: 1) recommend ideal storage conditions over a given period of time, 2) to determine the acceptability and assimilation of encapsulated materials in animals and 3) to ascertain the relative stability of the encapsulated materials.

Quality Assurance

The quality assurance/quality control (QA/QC) analyses were conducted to determine the chemical load, the purity profile, and indices of relative stability. In order to assess the quality of the microencapsulated fish oil and n-3 concentrates, a 12-month storage study was performed on the microencapsulated corn oil, vacuum-deodorized fish oil and ethyl ester. With the intent that ideal storage conditions can be recommended, these comparisons were performed at three temperatures (25°C, 5°C, and -20°C). A storage shelf life study conducted at these temperatures will provide information to recommend guidelines for the storage and shelf-life of the microencapsulated products for research studies. The sampling regime consisted of analyzing one bottle of each microencapsulated BTM stored at 5°C for lipid and fatty acid profile and peroxide value. The BTMs stored at -20°C, 5°C, and 25°C were subjected to the following analyses at 0, 6, and 12 months: lipid and fatty acid profile, peroxide value, sensory attributes, and antioxidant concentration.

The analytical methods utilized in this study are described in the QA/QC Manual for the BTM[5]. Both the intact microencapsulated material and lipid obtained by a modified Bligh and Dyer[15] extraction of the material were analyzed for fatty acid/ethyl ester composition. The extracted lipid comprised the residual nonencapsulated lipid and 80-90% of the encapsulated lipid. For analysis of the intact material, a known amount of internal standard (23:0 methyl ester or ethyl ester) was added to approximately 200 mg of the microencapsulated material. Fatty acid methyl esters (FAME) were prepared for the fish oil and corn oil products using the procedure given in the manual for the preparation of FAME (adjusting the amounts of reagents to accommodate the larger sample). Ethyl esters were recovered quantitatively in hot (100°C) methanol and extracted into iso-octane. Lipids recovered in this manner were used for calculation of load. Although the Bligh and Dyer[15] extraction was not effective in recovering the total microencapsulated lipid, the relative amounts of the individual recovered fatty acids/esters were nearly identical to those obtained from analyses of the intact materials. It was therefore assumed that this extracted lipid was representative of the total and suitable for the determination of lipid composition, peroxide values, cholesterol, and tocopherols. A hexane extraction was utilized for the determination of residual non-encapsulated lipid. Moisture was determined gravimetrically. TBA was determined by the method of Farladgis et. al.[16] for selected samples to provide supporting evidence for the level of lipid oxidation by the elevated peroxide values.

In terms of the QA/QC parameters measured over the 12 month time period, an overall analytical assessment would indicate that laboratory B produced a superior microencapsulated fish oil, ethyl ester, and corn oil product. Appendices 7a, 7b, and 7c displays the chemical analyses for the microencapsulated test materials produced by two different laboratories. The analyses indicate a decrease in the tocopherol concentration, both alpha- and gamma-, over the 12 month period, particularly for the ester capsules produced by company A. A small decrease in the tocopherol concentration occurred after processing but the major changes were evident when measured at the 6 and 12 month interval. It should be noted that while gamma-tocopherol was relatively stable during processing, as indicated by analyses at time 0, the alpha-tocopherol decreased. This apparently had a significant effect on the product produced by laboratory A. There was no significant decrease in gamma-tocopherol for any of the products produced by laboratory B. There were no observable changes in tocopherol concentration in the corn oil microcapsules. The percentage loss of EPA and DHA indicate that a greater decrease in both EPA and DHA for the fish oil as well as the ester occurred in the microcapsules produced by laboratory A. These losses are evident for the initial loss due to processing as well as the loss occurring over the storage time.

While the analyses shown in Appendices 7a, 7b, and 7c support the conclusion that laboratory B produced a superior microcapsules of the test materials, the reasons why are not so evident. There are three possible areas that may account for the differences in the performance of these microcapsules. First, the matrix is very different for these two microcapsules which may have signifcant implications in protecting the stability of the capsules. The gelatin matrix used by laboratory B would seem to infer less oxygen permeability to the capsule shell. The inherent characteristic of capsule permeability may also be coupled with the substantial decrease in the tocopherol

concentration found in capsules produced by laboratory A, especially for the ester. The decrease in gamma-tocopherol was 50% for the ester and 24% for the fish oil while the alpha-tocopherol decreased 85% for the ester and 25% for the fish oil. Without antioxidant protection afforded by the tocopherols (and perhaps also TBHQ which was not measured), conditions were favorable for the microcapsules produced by laboratory A to undergo oxidation. Second, the size of the capsules may be important in the relative stability and permeability of the products. Scanning electron microscopy revealed that the capsules produced by laboratory A averaged 50u while those produced by laboratory B averaged 200u. The larger sized microcapsules produced by laboratory B may have afforded better protection against oxidation. Third, the microencapsulation process obviously has an important role in producing a high quality product. An important aspect in producing and maintaining a high quality microencapsulated product is the initial production step. It is critial to the integrity of the product that the initial processing does not initiate oxidative reactions that would compromise the product. Although time 0 analyses indicate that oxidation was initiated during the processing step by both laboratories, the microcapsules produced by laboratory B had significantly less oxidation which would indicate a superior process.

The temperature storage studies[11] demonstrated that the temperature had little effect on the microcapsules produced by laboratory B, while having significant effects on those produced by laboratory A. Relatively small increases occurred in peroxide values for the fish oil and ester microcapsules produced by laboratory B at the higher temperature of 23° C compared to 5° C and -20° C. On the other hand, the lower temperature of -20° C afforded significant protection against oxidation as measured by peroxide value for the microcapsules produced by laboratory A.

Basically, the process of microencapsulation involves a number of parameters that need to be considered when microencapsulating a particular product. Some of the more important of these are: the physiocochemical requirements for microcapsules, the encapsulation process itself, and the physiological constraints. The physicochemcial requirements include uniform particle size, consistent chemical load, chemical integrity, low shell permeability, free flowing, coating durability, and a long shelf life. These parameters will be determined by the physical/chemical characteristics of the product (volatility, oxidizable, reactivity, and physical state). The encapsulation process needs to incorporate the most appropriate shell composition and have compatibility with the product. The physiological constraints frequently include bioavailability, digestibility of the coating, and rapid release. The physiochemical and pharmcological properties of drugs/chemicals and the intended use of their products will tend to dictate how they should be microencapsulated.

Recently, we have performed a limited chemical assessment on fish oil and n-3 ethyl ester concentrate microencapsulated by a third laboratory. The microencapsulated test material has maintained a low peroxide value (<3.0 meq/kg) over a 6 month storage period at 5° C. Results at 9 months indicate an increase in peroxide value to 6 meq/kg for the fish oil to 42.5 meq/kg for the n-3 ethyl ester concentrate. The composition of the microencapsules are: 25% test material, 25% starch, 22% gelatin, 19% sucrose, and minor amounts of

ascorbic acid, calcium ascorbate, and tricalcium phosphate. It therefore seems likely that a high quality microencapsulated test material can be produced having good stability characteristics.

Bioavailability Tests - Animal F eding Trials

The short term animal feeding studies were designed to investigate the acceptability and assimilation of the encapsulated fish oil and ester stored at 5°C. The animal feeding trials were conducted in parallel with the QA/QC storage stability study, thereby, allowing changes observed in the acceptability and assimilation studies to be correlated with changes detected in the quality of the microcapsules.

Study A

Study A conducted by Dr. Norberta Schoene (USDA, Lipid Nutrition Lab, Beltsville, MD) was designed to test the microencapsulated test materials produced by laboratories A and B and to assess the performance of the diets under various feeding regimes. Diets were prepared with microencapsulated fish oil, corn oil, n-3 ethyl ester concentrate prepared by laboratory A and B and compared to diets containing liquid corn oil and fish oil. Rats received the diets for a period of 8 days using the following protocols: Male Wistar rats (n=8, 250 g) were fed the diets for one week. Fresh food stored at 5°C was given to the animals daily. The results indicate that there was no difference in weight gain among the dietary treatments. The liver as percent body weight was also the same for all treatment groups. The fatty acid composition of plasma and platelet phospholipids were the same for the microencapsulated and liquid fish oil as well as for the different forms of n-3 ethyl ester concentrate.

Study B

Study B was conducted by Dr. Norman Salem (Alcohol, Drug Abuse and Mental Health Administration, Analytical Chemistry-Laboratory of Clinical Studies) to determine the palatability and the bioavailability of the microencapsulated fish oil and n-3 concentrate. The experiment was designed to allow direct comparison of the efficacy of various formulations of n-3 fatty acids with respect to their influence in altering the fatty acyl distribution in plasma and tissues. The following four treatments were administered to rats during a 10 day period: 1) 9% olive oil by gavage, 2) 9% menhaden oil by gavage, 3) 9% microencapsulated menhaden oil (Laboratory A) in the diet, 4) 3.63% microencapsulated n-3 ester concentrate (Laboratory A) in the diet and 5.36% olive oil by gavage. The intent of the study was to feed groups of rats (n=5) the same amount of fish oil either in an microencapsulated form or as an oil. The total amount of n-3 fatty acid was balanced between the fish oil and n-3 ester group and the total fat intake controlled by the addition of olive oil. Results indicated that the microencapsulated material had similar efficacy as the liquid fish oil since the fatty acid composition of the plasma and tissue of the rats receiving both treatments were the same. The results of this study concluded that the digestion and bioavailability of microencapsulated and intubated fish oil were similar. The n-3 ethyl ester also had similar efficacy in affecting rat plasma fatty acid content as the liquid fish oil.

The animal feeding studies demonstrated that the microcapsules are a good dosage delivery form for feeding n-3 fatty acids to animals. The basis of this assessment is threefold: 1) the rats readily ate the purified diet containing fish oil or ethyl ester microcapsules, 2) the rats gained weight at the same rate as those fed corn oil or liquid menhaden oil, and 3) the microcapsules are a convenient dosage form. Results from Dr. Salem's study show the efficacy of the microcapsules is the same as intubated fish oil with respect to altering the plasma fatty acid compositon. In addition, the ethyl ester microcapsules, when fed in a dose that contains the same amount of total n-3, produced changes in rat plasma fatty acids that were about the same as those in either the fish oil or microencapsulated fish oil fed animals.

Although physiological parameters indicate that the microcapsules of fish oil and ester concentrates are comparable to the liquid forms of the test materials, the chemical analyses demonstrate distinct differences between the them. The peroxide value of microcapsules produced by laboratories A and B increased over the length of the study, although microcapsules produced by Laboratory A experienced a much greater increase. Microcapsules produced by Laboratory B were superior in terms of stability as measured by its lower peroxide value, smaller losses of EPA and DHA, and greater retention of antioxidants. However, the stability of microcapsules produced by both laboratories are certainly not acceptable over a 12 month period. Since the feeding studies on the microencapsulated fish oil and ethyl ester were performed on capsules stored for approximately 5 months peroxide values for the n-3 ethyl ester and VDFO of 100 meq/kg and 41 meq/kg, and 210 meq/kg and 41 meq/kg, for the Laboratory A and Laboratory B microcapsules, respectively, were used. While no toxic effects were evident in these trials and the growth rate of animals receiving the treatments were similar to the corn oil microcapsules and liquid fish oil, the feeding of products with high peroxide values poses a real concern, especially in long feeding trials. Although these two types of microcapsules did not demonstrate good stability, it is possible to produce microencapsulated fish oil and n-3 ethyl ester concentrate with good stability characteristics as indicated by the recent encapsulation of the test materials by a third manufacturer.

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FORMULATION OF CONTROL BULK OIL

The objective of the NOAA Fish Oil Biomedical Test Material Program is to provide researchers quality assured test materials needed to develop a thorough understanding of the biochemical interactions of n-3 fatty acids. In concert with this objective, NOAA will offer to quality assure the control bulk oil purchased by the researcher. Samples of corn oil or other control oil purchased for use as control bulk oil should be sent to the NOAA Charleston Laboratory prior to the addition of antioxidants. Please send a 5 ml sample of corn oil sealed under nitrogen in an amber, glass or plastic container. The Charleston Laboratory will analyze the oil for antioxidants so that the tocopherols and TBHQ can be balanced with the fish oil. After addition of the antioxidants, mix thoroughly and send another 5 ml sample to verify correct addition of antioxidants. Below is listed a source of corn oil, and antioxidant recommended levels of antioxidant addition, and storage of the test material.

Source:

Corn Oil:	Teklad[1] P.O. Box 4220 Madison, Wisconsin 53711 (608)274-9008
Antioxidants:	Eastman Chemical Products P.O. Box 431 Kingsport, Tennessee 37662 (800)327-8626

Antioxidants	g antioxidant/g preparation
Tenox 20A	
20% tertiary butylhydroquinone (TBHQ)	0.20
(3% citric acid, 30% corn oil, 32% glyceryl monooleate, 15% propylene glycol)	
Vitamin E-5-67 (672 mg/g alpha tocopherol)	0.67
Tenox GT-1 (50% tocopherols; 50% edible soy oil)	
60-65% gamma-tocopherol; 300-325 mg	0.30-0.32
15% alpha-tocopherol; 75 mg	0.075
15% delta-tocopherol; 75 mg	0.075

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Balancing of antioxidants

The antioxidants should be balanced as described below:

Fish Oil (g/kg)

	present	need	total
alpha-tocopherol	-	1.0	1.0
gamma-tocopherol	-	1.0	1.0
TBHQ	-	0.2	0.2

Corn Oil (g/kg)

	present	need	total
alpha-tocopherol	0.2	0.8	1.0
gamma-tocopherol	0.6	0.4	1.0
TBHQ	-	0.2	0.2

The concentration of antioxidants added to the fish oil by the NMFS appear below:

Fish Oil

	present	add (g/kg)	final amounts (g/kg)		
			alpha	gamma	TBHQ
alpha-tocopherol	-	Kodak 567 (1.15)	0.77	-	-
gamma-tocopherol	-	Tenox GT-1 (3.1)	0.23	1.01	-
TBHQ	-	Tenox 20A (1.0)	-	-	0.2
		Total	1.00	1.01	0.2

The antioxidants should be added to the corn oil in the concentrations described below:

Corn Oil

	present	add (g/kg)	final amounts (g/kg)		
			alpha	gamma	TBHQ
alpha-tocopherol	0.2 g/kg	Kodak 567 (1.06)	0.71	-	-
gamma-tocopherol	0.6 g/kg	Tenox GT-1 (1.2)	0.09	0.4	-
TBHQ	-	Tenox 20A (1)	-	-	0.2
		Total	0.8	0.4	0.2

Storage: The container should be blanketed with nitrogen prior to resealing and stored at 5°C. Bulk control oil should be stored and protected in the same manner as the fish oil.

APPENDIX 2A

Requirements for Formal Request Concerning Exportation of an Unapproved New Drug

In accordance with U.S. Food and Drug Administration regulations concerning exportation of an unapproved new drug certain criteria must be met in order for the U.S. Department of Commerce to release Biomedical Test Materials (henceforth called drug) for shipment to a foreign country.

A formal request by an authorized national government official of your country must be made to the U.S. Food and Drug Administration's International Affairs Staff and copy forwarded to the Charleston Laboratory.

This formal request, on official national government letterhead of your country, must include the following items:

1. That your government has adequate information about the drug and its proposed investigational use.

2. Stated acknowledgement that the drug will be used for investigational use only.

3. A statement that your government is satisfied the drug may legally be used by you, the consignee.

4. An indication of the source (U.S. Department of Commerce, NOAA, National Marine Fisheries Service, Charleston Laboratory, P.O. Box 12607, Charleston, SC 29412). Please reference Drug Master File (DMF) #7439 of the U.S. Food and Drug Administration concerning the drug.

5. An indication of the quantity of the drug to be shipped, as well as the expected frequency of shipments.

§ 312.110 Import and export requirements.

(a) *Imports.* An investigational new drug offered for import into the United States complies with the requirements of this part if it is subject to an IND that is in effect for it under § 312.40 and: (1) The consignee in the United States is the sponsor of the IND; (2) the consignee is a qualified investigator named in the IND; or (3) the consignee is the domestic agent of a foreign sponsor, is responsible for the control and distribution of the investigational drug, and the IND identifies the consignee and describes what, if any, actions the consignee will take with respect to the investigational drug.

(b) *Exports.* An investigational new drug intended for export from the United States complies with the requirements of this part as follows:

(1) If an IND is in effect for the drug under § 312.40 and each person who receives the drug is an investigator named in the application; or

(2) If FDA authorizes shipment of the drug for use in a clinical investigation. Authorization may be obtained as follows:

(i) Through submission to the International Affairs Staff (HFY-50), Associate Commissioner for Health Affairs, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, of a written request from the person that seeks to export the drug. A request must provide adequate information about the drug to satisfy FDA that the drug is appropriate for the proposed investigational use in humans, that the drug will be used for investigational purposes only, and that the drug may be legally used by that consignee in the importing country for the proposed investigational use. The request shall specify the quantity of the drug to be shipped per shipment and the frequency of expected shipments. If FDA authorizes exportation under this paragraph, the agency shall concurrently notify the government of the importing country of such authorization.

(ii) Through submission to the International Affairs Staff (HFY-50), Associate Commissioner for Health Affairs, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, of a formal request from an authorized official of the government of the country to which the drug is proposed to be shipped. A request must specify that the foreign government has adequate information about the drug and the proposed investigational use, that the drug will be used for investigational purposes only, and that the foreign government is satisfied that the drug may legally be used by the intended consignee in that country. Such a request shall specify the quantity of drug to be shipped per shipment and the frequency of expected shipments.

(iii) Authorization to export an investigational drug under paragraph (b)(2)(i) or (ii) of this section may be revoked by FDA if the agency finds that the conditions underlying its authorization are not longer met.

(3) This paragraph applies only where the drug is to be used for the purpose of clinical investigation.

(4) This paragraph does not apply to the export of an antibiotic drug product shipped in accordance with the provisions of section 801(d) of the act.

(5) This paragraph does not apply to the export of new drugs (including biological products) approved for export under section 802 of the act or section 351(h)(1)(A) of the Public Health Service Act.

(Collection of information requirements approved by the Office of Management and Budget under number 0910-0014)

[52 FR 8831, Mar. 19, 1987, as amended at 52 FR 23031, June 17, 1987]

APPENDIX 3A. STORAGE STABILITY OF BULK PACKED VACUUM DEODORIZED FISH OIL WITH ANTIOXIDANT[1] STORED AT - 40°C AND 5°C IN AMBER, LOW DENSITY POLYETHYLENE[2] (LDPE) AND HIGH DENSITY POLYETHYLENE[3] (HDPE) CONTAINERS.

TIME (months)	TREATMENT	PV	AV	a-Toc	g-Toc	EPA	DHA	TOT n-3
		0.43	34.7	0.6	0.9	134	85	298
3	HDPE[2] (-40°C)	0.44	-	-	-	-	-	-
	HDPE[2] (5°C)	2.75	-	-	-	-	-	-
	HDPE[3] (5°C)	3.06	-	-	-	-	-	-
6	HDPE[2] (-40°C)	0.43	33.8	0.8	1.1	134	85	298
	HDPE[2] (5°C)	9.12	33.0	0.8	1.1	133	84	295
	HDPE[3] (5°C)	10.08	33.4	0.8	1.1	134	85	297
9	HDPE[2] (-40°C)	0.42	-	-	-	-	-	-
	HDPE[2] (5°C)	11.43	-	-	-	-	-	-
	HDPE[3] (5°C)	15.71	-	-	-	-	-	-
12	HDPE[2] (-40°C)	0.26	35.8	0.8	1.1	130	81	289
	HDPE[2] (5oC)	16.37	37.1	0.8	1.1	129	81	288
	HDPE[3] (5°C)	22.62	37.4	0.8	1.1	128	80	284

[1] Antioxidants added to the vacuum-deodorized fish oil are: 1.0mg/g alpha-tocopherol; 1.0 mg/g gamma-tocopherol; and 0.02% TBHQ.

[2] HDPE containers are 125 ml amber #2009-004; wall thickness 0.288", density 0.95; manufactured by Nalgene, Rochester, NY.

[3] HDPE containers are 120 ml amber #41872; wall thickness 0.293", density 0.94; manufactured by Alpha Plastics, St. Louis, MO.

APPENDIX 3B. STORAGE STABILITY OF BULK PACKED VACUUM DEODORIZED FISH OIL WITHOUT ANTIOXIDANT[1] STORED IN THREE TYPES OF BOTTLES AT -4°C AND 5°C.

TIME (months)	TREATMENT	PV	AV	a-Toc	g-Toc	EPA	DHA	TOT n-3
0		0.66	36.5	<0.05	<0.05	144	88	314
3	HDPE[2] (-40°C)							
	HDPE[2] (5°C)	4.09	-	-	-	-	-	-
	HDPE[3] (5°C)	8.53	-	-	-	-	-	-
	GLASS[4] (5°C)	1.35	-	-	-	-	-	-
6	HDPE[2] (-40°C)	0.87	36.9	-	-	134	85	299
	HDPE[2] (5°C)	7.86	35.8	-	-	132	84	295
	HDPE[3] (5°C)	11.00	38.9	-	-	134	85	298
	GLASS[4] (5°C)	1.77	33.9	-	-	133	84	297
9	HDPE[2] (-40°C)	-0.97	-	-	-	-	-	-
	HDPE[2] (5°C)	-9.25	-	-	-	-	-	-
	HDPE[3] (5°C)	-11.82	-	-	-	-	-	-
	GLASS[4] (5°C)	-0.84	-	-	-	-	-	-
12	HDPE[2] (-40°C)	0.81	38.5	-	-	131	83	292
	HDPE[2] (5°C)	11.83	41.6	-	-	130	82	289
	HDPE[3] (5°C)	15.46	43.4	-	-	129	82	289
	GLASS[4] (5°C)	1.95	38.2	-	-	129	82	288

[1] Antioxidants (a-Toco, alpha-tocopherol; g-Toco, gamma-tocopherol; and tertiary butylhydroquinone) were not added to the vacuum-deodorized fish oil.

[2] HDPE - high density, polyethylene, amber containers (125 ml #2009-004; wall thickness 0.288", density 0.95; manufactured by Nalgene, Rochester, NY).

[3] HDPE - high density, polyethylene, amber containers (100 ml #41872; wall thickness 0.293", density 0.94; manufactured by Alpha Plastics, St. Louis, MO).

[4] Glass containers are 120 ml amber containers with teflon cap liners (#7919; manufactured by All-Pak, Pittsburgh, PA).

APPENDIX 3C. STORAGE STABILITY OF BULK PACKED n-3 ETHYL ESTER CONCENTRATE STORED IN HDPE[1] CONTAINERS 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

[1] HDPE - High density polyethylene, amber, containers (Nalgene, Rochester, NY)

APPENDIX 3D. STORAGE STABILITY OF SOFTGEL CAPSULES OF VACUUM DEODORIZED FISH OIL.

	MONTHS STORED		
ANALYSIS TYPE	0	12	24*
TRIGLYCERIDE, %			
EPA, mg/g	136	140	
DHA, mg/g	81	84	
TOTAL n-3, mg/g	308	305	
FREE FATTY ACIDS, %			
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	
MOISTURE, ug/g			
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	0.17	
BURNT	0	0.23	

* - Data to be collected 12/90.

APPENDIX 3E. STORAGE STABILITY OF SOFTGEL CAPSULES OF N-3 ETHYL ESTER CONCENTRATE STORED AT 5° C

	MONTHS STORED				
ANALYSIS TYPE	0	3	6	9	12
ESTER, %	919	906	899	-	906
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	-	0.99
g-TOCOPHEROL, mg/g	2.1	1.67	1.62	-	1.00
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
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	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

APPENDIX 4 EXPERIMENTAL SEMIPURIFIED DIETS

The omega-3 polyunsaturated fatty acids (PUFA) present in fish oil test materials have 5-6 double bonds and are prone to undergo oxidative reactions when exposed to air at room temperature. Thus, special precautions have to be taken to maintain consistent quality for proper evaluation of omega-3 PUFA test materials.

Formulation:

In devising nutritionally adequate diets that can be used for rats and mice, it is important to eliminate, as much as possible, the variables that commercial diets from unrefined natural products would normally introduce. Therefore, the percent composition of experimental diets should be based on guidelines set by the American Institute of Nutrition Ad Hoc committee (1-2).

There are three basic methods to formulate a high-fat diet: dilution, substitution, or isonutrient replacement. Particular attention should be given to control of the nutrient density of the diets. Fat should not be used as a diluent of a premixed ration unless that ration has been especially formulated to contain an excess concentration of vitamins, minerals, and protein. When different concentrations of fat are used in the diet, the other nutrients should be adjusted on a calorie basis to avoid dilution of the nutrients (3). Similar adjustments should be made if different concentrations of non-nutritive fiber are included in the diet. Young rodents adjust their food intake to equalize energy intake. Animals that do not regulate their own intake (i.e., swine) should be fed a measured quantity of food rather than be allowed to eat ad libitum.

When testing different types of fat sources, the composition of fatty acids, vitamins, minerals, antioxidants, and other ingredients should be determined. This information can be obtained from commercial suppliers in most instances, but its accuracy should be checked. Dietary fat should contain an adequate source of essential fatty acids (EFA). If only fish oil were used, the potential for artifactual results exists, since the diet might be low in omega-6 EFA.

It is recommended that the type and amount of antioxidant in other oil sources be adjusted to match the composition of the fish oil test material. All fish oils contain only alpha-tocopherol, the most bipotent isomer. Vegetable oils contain various mixtures of isomers, and this should be taken into consideration in balancing the ratio.

Preparation:

All experimental semipurified diets and test materials should be stored at 4°C. Where possible, the fats and oils should be stored under an atmosphere of nitrogen or argon. Based upon the experiences of a number of investigators, it is recommended that the experimental diets be prepared twice weekly and stored in air-tight, non-plastic containers (or containers lined with heavy-duty aluminum foil) at 4°C, or at -70°C where possible, in the dark. The material in the feed cups must be replaced on a daily basis. In instances where the diet residue dries up in the feed cup, an alcohol swab should be used to wipe out the residue before adding fresh food.

Quality Control:

Fatty acid analysis of different fats and oils should be performed periodically. Analyses of experimental diets should be performed at the beginning and at various time intervals after the diet is prepared in the facilities available to the investigator. Specifically, changes in the total amount of omega-3 PUFA should be noted. Additionally, the diets should be monitored for perioxides by

methods suitable for PUFA with 2 or more double bonds (4, 5). It has been suggested by some investigators that food intake of rats and mice decreases rapidly when omega-3 PUFAs have oxidized in the experimental diet. Therefore, it would be useful to monitor food intake and body weight changes as good experimental practices.

Based upon overall findings and observations, each investigator should decide whether the diets need to be prepared on a daily, bi-weekly, or weekly basis.

REFERENCES

1. Bieri, J.G. et al. J. Nutr. 107:1340-1348, 1977.
2. Bieri, J.G. et al. J. Nutr. 110:1726, 1980.
3. J. AOCS 42:903, 1965.
4. Peroxide Value. A.O.C.S. Official Method Cd 8-53.
5. Sidwell, C.G. et al. J. Am. Chem. Soc. 31:603-606, 1954.

APPENDIX 5A. 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
Id ntification	: 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
H avy 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.
Ref rences	: Vendor Protocol

APPENDIX 5B. 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 tast . 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.
Ref rences	: U.S. Pharmacopeia XXI, p.464

* Designates official USP XXI specification

APPENDIX 6. 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"

APPENDIX 7A. CHEMICAL COMPOSITION AND QUALITY DATA ON MICROENCAPSULATED VACUUM-DEODORIZED FISH OIL PRODUCED BY TWO LABORATORIES.

CHEMICAL ANALYSES	BEFORE	TIME 0 LAB A	TIME 0 LAB B	6 MO LAB A	6 MO LAB B	12 MO LAB A	12 MO LAB B
Load (%)		42	52				
Hexane Wash (%)		7	26				
Moisture (%)		5.2	0.6				
Triglyceride (%)		94.2	96.5	88.5		80	91
EPA (mg/g oil)	127	118	119				
EPA (mg/g material)		49	61	46	61	34	60
DHA (mg/g oil)	75	70	71				
DHA (mg/g material)		29	37	27	37	19	35
Total n-3 (mg/g oil	260	259	260				
Total n-3 (mg/g material)		108	134	100	138	73	- 132
Cholesterol (mg/g)	1.3	1.3	1.31	1.2	1.2	-	1.3
a-tocopherol (mg/g oil)	1.5	0.98	1.24	0.7	1.1	-	1.1
g-tocopherol (mg/g oil)	1.1	1.15	1.09	0.9	1.0	-	1.1
Peroxide value (meq/kg)	1.5	31	18.8	142	34.4	294	56.6
TBA (umol/kg material)		30					

APPENDIX 7B. CHEMICAL COMPOSITION AND QUALITY DATA ON MICROENCAPSULATED CORN OIL PRODUCED BY TWO LABORATORIES.

CHEMICAL ANALYSES	BEFORE	TIME 0 LAB A	TIME 0 LAB B	6 MO LAB A	6 MO LAB B	12 MO LAB A	12 MO LAB B
Load (%)		43	54				
Hexane Wash (%)		8	29				
Moisture (%)		5.8	0.2				
Triglyceride (%)		96.7	97.7				
EPA (mg/g oil)							
EPA (mg/g material)							
DHA (mg/g oil)							
DHA (mg/g material)							
Total n-3 (mg/g oil		8.3	6.7				
Total n-3 (mg/g material)		3.5	3.6	3.2	3.3	3.7	4.1
Cholesterol (mg/g)							
a-tocopherol (mg/g oil)	0.31	0.44	0.85	0.7	0.8		
g-tocopherol (mg/g oil)	0.94	0.86	0.53	0.9	0.5		
Peroxide value (meq/kg)	1.5	14.4	5.78	24.5	7.5	42.7	10.0
TBA (umol/kg material)		30					

APPENDIX 7C. CHEMICAL COMPOSITION AND QUALITY DATA ON MICROENCAPSULATED N-3 ETHYL ESTER CONCENTRATE PRODUCED BY TWO LABORATORIES.

CHEMICAL ANALYSES	BEFORE	TIME 0 LAB A	TIME 0 LAB B	6 MOS. LAB A	6 MOS. LAB B	12 MOS. LAB A	12 MONTHS LAB B
Load (%)		44	49				
Hexane Wash (%)		1.3	26				
Moisture (%)		5	0.9				
Triglyceride (%)		92.6	93.6				91
EPA (mg/g oil)	430	411	431				
EPA (mg/g material)		180	209	141		106	184
DHA (mg/g oil)	200	185	197				
DHA (mg/g material)		81	95	64		48	87
Total n-3 (mg/g oil	750	689					
Total n-3 (mg/g material)		280		242	3.3	178	310
Cholesterol (mg/g)	1.0	1.02	1.02	1		-	-
a-tocopherol (mg/g oil)	1.3	1.02	1.13	0.2	0.8	-	-
g-tocopherol (mg/g oil)	1.0	1.07	1.04	0.5	0.5	-	-
Peroxide value (meq/kg)	6.28	52.8	27.7	236.8	42.6	280.4	59.7
TBA (umol/kg material)		73.6					