NOAA TECHNICAL MEMORANDUM NMFS—SEFC—211

BIOMEDICAL TEST MATERIALS PROGRAM: ANALYTICAL METHODS FOR THE QUALITY ASSURANCE OF FISH OIL

Frances M. Van Dolah
Sylvia B. Galloway
Editors

November 1988

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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U.S. DEPARTMENT OF COMMERCE
C. William Verity, Secretary
NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
William E. Evans, Administrator
NATIONAL MARINE FISHERIES SERVICE
James W. Brennen, Assistant Administrator for Fisheries

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1 INTRODUCTION

The compilation of methods contained in this manual represent the standard methods utilized by the NMFS FISH OIL BIOMEDICAL TEST MATERIALS (BTM) PROGRAM to conduct quality assurance and quality control. The Fish Oil Biomedical Test Materials Program (BTM) was initiated in 1986 by the signing of a memorandum of understanding (MOU) between the National Oceanic and Atmospheric Administration (NOAA) and the National Institutes of Health (NIH)/ Alcohol, Drug Abuse and Mental Health Administration (ADAMHA). Under the MOU it was agreed that the Charleston Laboratory of NMFS would provide a long-term consistent supply of test materials in order to facilitate the evaluation of the role of omega-3 fatty acids in health and disease. A sub-committee of the Nutrition Coordinating Committee of NIH, the Fish Oil Test Materials Advisory Committee (FOTMAC), provides the review and approval mechanism for the distribution of quality assured/quality controlled test materials to researchers. The applicants are researchers who are funded by NIH, ADAMHA, and other research organizations.

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

The Charleston Laboratory currently produces vacuum-deodorized menhaden oil (VDMO) and omega-3 PUFA ethyl ester concentrates (>85%), provided in bulk form or gelatin encapsulated. A quality assurance report accompanies each lot of test materials shipped from the laboratory detailing the chemical composition, oxidation and degradation products, and low levels of contaminants which may be present in the products. This information is used by the investigators to alert them of any minor components which may possess an physiological activity in their particular experimental system which may impact the interpretation of their results.

Many of the methods used are official methods of the Association of Official Analytical Chemists (AOAC) or American Oil Chemists Society (AOCS). Many of the official methods were developed for the analysis of vegetable oils and have been modified for analysis of fish oil. In some cases, current technology far exceeds the official method, and the program has utilized the newer technique. All methods are described in detail, and include information on results of collaborative efforts using the method.

The manual contains nine sections including the general introduction. Each section of the manual deals with specific types of analyses: lipid characterization, sterols, fatty acid oxidation products, organics, metals, moisture, sensory attributes, and bacteria. Each method is described using the following outline: introduction, principle, apparatus, reagents, preparation of standards and samples, determination, calculation, precision, notes, and references.

This volume is intended as an interim edition. Some methods useful for assessing quality of the BTMs are currently being developed and will be included in a more complete manual to be published at a later date. Methods currently being developed at the Charleston Laboratory include the analysis of PCBs and pesticides in esters, analysis of trace elements in oils and esters by graphite furnace atomic absorption spectroscopy, conjugated fatty acids, polar oxidation products, and measurement of volatiles by head space analysis. *Since this is intended as an interim edition of the quality assurance methods manual we would like to ask users of this volume for any editorial comments they may have once the volume has been used.*

The editors wish to acknowledge the following contributors for their invaluable assistance in compiling the methods presented in this volume: Gloria T. Seaborn, Jeanne D. Joseph, Cheryl R. Sivertsen, Janet A. Gooch, Patsy P. Bell, Vijay A. Koli, and Gregory B. Mitchum.

The mention of trade names is for informational purposes and is not intended as an endorsement of the product(s) by the National Marine Fisheries Service.

2 LIPID CHARACTERIZATION

2.1 LIPID CLASS PROFILE

INTRODUCTION

Thin layer chromatography (TLC) is utilized by the BTM Program to qualitatively monitor product composition through all phases of production and to assure purity prior to product distribution. TLC and gas chromatography are the chromatographic methods most frequently used for lipid separation (1). TLC is particularly effective for the separation of intact complex lipids and neutral lipids (2,3). Although no official method exists for the examination of lipid composition by TLC, many standard methods employ TLC as a means of isolation and purification of sample components for subsequent analysis.

PRINCIPLE

Fats, oils, waxes and other neutral lipids are separated by adsorption chromatography on silica gel G plates. Lipid samples and purified standards in chloroform are applied as discrete spots, 1.5-2.0 cm from the lower edge of the plate. The plate is then transferred to a developing tank containing 100 ml of the eluting solvent (approximately 1 cm in depth). As the solvent moves up the plate by capillary action, the lipid components are separated according to their affinity for the adsorbent silica gel. For neutral lipids, the developing solvent is usually a mixture of petroleum ether and diethyl ether. When the solvent front is near the top of the plate, the plate is removed from the tank, air dried, and sprayed with an indicating reagent.

APPARATUS

* TLC plates, 20 cm x 20 cm, precoated silica gel G, with preadsorbent layer (J.T. Baker Si250F PA or equivalent)
* Syringe for sample application (Hamilton #701N or equivalent)
* Dessicator for storing plates (Alltech Assoc., Inc)
* Glass chromatography tank with glass cover (Supelco)
* Adsorbent paper for lining tanks (Alltech Assoc., Inc)
* Spotting guide (Supelco)
* Drying oven (GCA/Precision Scientific)
* Chromist sprayer (Gelman Sciences)
* TLC spray box (Alltech Assoc., Inc)

REAGENTS

* Hexane: diethyl ether: glacial acetic acid (80:20:1 v/v/v) (ACS reagent grade)
* Chloroform (ACS reagent grade)
* Phosphomolybdic acid (reagent grade), 5% (w/v) in 95% ethanol, stored in amber bottle
* Ethanol, 95% (reagent grade)
* Mixed TLC standards (steryl esters, methyl esters, triacylglycerol, free fatty acid, cholesterol, mono- and di-acylglycerol, Nu Chek Prep catalog Nos. 18-1-A and 18-4-A), in sealed ampules.

PREPARATION OF STANDARDS & SAMPLES

1. **Standards:** Standards should be stored in glass ampules at -20°C until opened. After opening, lipids should be stored in chloroform in nitrogen-purged vials, fitted with crimp or screw caps with teflon-lined septa, at -20°C.
2. **Samples:** Dissolve each lipid sample in chloroform to give a concentration of 150-200 *ug* lipid/*ul*.

DETERMINATION

1. Line the chromatography tank on three sides with adsorbent paper. Add 100 ml solvent, cover (Note 1) and allow 1-2 h for equilibration.
2. Remove TLC plate from dessicator immediately prior to spotting and place on a clean, dust-free surface (Notes 2 and 3).
3. Position the spotting guide so that the sample may be applied to the plate approximately 0.5 cm below the top of the preadsorbent area.
4. Spot 1 and 2 *ul* aliquots of samples and standards. Allow the chloroform to evaporate by passing clean, dry nitrogen over the surface of the plate.
5. Score the coating material approximately 2 cm from the top of the plate.
6. Place the plate in the developing tank to allow the solvent to ascend to the score line (30-40 min). Remove the plate from the tank and allow the solvent to evaporate.
7. Place the plate in the spray box and spray evenly with phosphomolybdic acid.
8. Transfer plate to 110°C drying oven for 5 min, or until best spot definition is achieved.

CALCULATION

TLC is utilized by the QA/QC Project as a qualitative method of lipid identification. Lipid components are identified by comparison with migration distance of standards spotted on the same plate. Plates may be photographed or photocopied for permanent data storage.

NOTES

1. A weight should be placed on the glass lid to prevent evaporation of solvent during development.
2. More consistent results are obtained if plates are stored in a dessicator.
3. Disposable gloves should be worn when handling plates to prevent contamination of the plates with skin lipids.

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2.2 FATTY ACID COMPOSITION

INTRODUCTION

The analysis of the esters of fatty acids has been traditionally conducted by gas liquid chromatography (GLC) with separation on packed columns. The method is published as an official AOAC method (#28.060-28.068, final action)(1). The preparation of methyl esters of fatty acids in oils and fats is also published in an official AOAC method (#28.055-28.059, final action) (2). Currently the method of choice for the analysis of marine oils is a refinement of these procedures utilizing state of the art wall-coated open-tubular (WCOT) capillary columns. The method utilized by the BTM Program for the routine analysis of EPA, DHA, and other fatty acids is a method developed at the Charleston Laboratory (3) and is very similar to methods used by others in the omega-3 research community.

For the analysis of marine lipids, WCOT (capillary) columns offer several distinct advantages over packed columns. Most positional and geometric isomers found in marine lipids, with C-12 to C-24 carbon chain lengths, may be separated in 30-50 min. Background noise is reduced relative to packed column chromatography, allowing detection of minor fatty acids. Quantitation of *trans* fatty acids chromatographed on capillary column may eliminate the need for additional analysis of *trans* fatty acid content. Recent advancements in instrumentation and column technology have largely eliminated the quantitation problems experienced in early capillary column analyses.

PRINCIPLE

An accurately weighed sample of oil is mixed with a known amount of internal standard and saponified with alcoholic NaOH. The soaps are reacted with boron trifluoride in methanol to produce the fatty acid methyl esters (FAME). The esters are extracted into isooctane using a salting out technique and are analyzed by GLC with flame ionization detection (FID). Separation is achieved on a polar fused silica capillary column. Fatty acid standards, prepared and chromatographed in the same manner as the samples, are used for identification and quantitation of the FAME.

APPARATUS

* Gas chromatograph (Hewlett Packard 5890 or equivalent), designed to accept capillary columns (0.22-0.32 mm ID), with an FID detector and an electronic integrator
* Polar flexible fused silica capillary column (0.22-0.32 mm ID) at least 30 m in length, DB225 cyanopropylphenyl (J and W Scientific) or Supelcowax -10-polyethylene glycol (Supelco)

* Centrifuge (Precision Centricone or equivalent) with rotor to accomodate 16x125 mm screw cap culture tubes and capable of at least 1300 rpm
* Nitrogen evaporator with water bath capable of maintaining 37°C (Meyers N-Evap or equivalent)
* Vortex mixer
* Pasteur pipettes, disposable
* Automatic positive displacement pipettor capable of delivering 1 ml ± 0.01 ml (Eppendorf repeater pipette No. 2226 000-6 or equivalent)
* Disposable culture tubes, 16x125 mm, with teflon lined-screw caps (Kimble #45066-A or equivalent)
* Temperature block modular heater, with block to accomodate 16x125 mm tubes and controlled temperature at 100 ± 2°C
* Hamilton syringe, 10 *u*l
* Moisture trap (Chrompak #7971 or equivalent)
* Oxygen trap (Chrompak #7970 or equivalent)

REAGENTS

* Isooctane (ACS reagent grade)
* Methanol, absolute (ACS reagent grade)
* Boron trifluoride, 12% in methanol (Supelco)
* Sodium hydroxide (reagent grade), 0.5N in methanol
* Sodium chloride (reagent grade), saturated aqueous solution
* Butylated hydroxytoluene (BHT), reagent grade
* Methyl tricosanoate, 99+% (Nu Chek Prep)
* Simple triacylglycerol standards for the major fatty acids to be determined. Methyl esters may be used for fatty acids for which triacylglycerol standards are not available (Nu-chek Prep, Supelco). (Note 1)
* Helium or hydrogen (99% pure or better), carrier gas
* Nitrogen, compressed, dried (for solvent removal and make-up gas)

PREPARATION OF STANDARDS AND SAMPLES

I. **Standards**

1. **Internal standard.** Weigh approximately 100mg (to the nearest 0.1 mg) methyl tricosanoate into a 100 ml volumetric flask and make to volume with isooctane containing 20 ug/ml BHT.

2. **Reference standards.** Weigh to nearest 0.1 mg into a volumetric flask and make to volume with isooctane. Cost and availability of the standards must be considered in determining weights and volumes used.

II. **Preparation of Esters**

1. Weigh approximately 25 mg (to the nearest 0.1 mg) of oil into a 16x125 mm screw-capped culture tube.
2. Add 1.0 ml methyl tricosanoate internal standard (IS). Mix thoroughly and evaporate to dryness under a stream of N_2 using a nitrogen evaporator.
3. Add 1.5 ml of 0.5N NaOH, cap tightly, vortex, and heat for 5 min at 100°C in heating block.
4. Cool with tap water.
5. Add one ampule (2 ml) of boron trifluoride; cap tightly, vortex, and return the tube to the heating block for 5 min.
6. Cool to 37°C in water bath, add 1 ml isooctane and vortex.
7. Add 3 ml saturated NaCl and extract into isooctane by agitating tube for approximately 1 min.
8. Centrifuge at 1300 rpm for approximately 2 min.
9. With a pasteur pipet, transfer the (upper) isooctane phase to a small vial containing anhydrous Na_2SO_4 (approximately a 2 mm layer).
10. Cap, shake, and allow to stand for approximately 20 min. Transfer to appropriate vial for GC analysis.

DETERMINATION

I. **Gas Chromatography System**

Injection System: The injection port should be fitted with an all-glass, split-injection line. The split ratio used is approximately 1:100. One *u*l of sample is injected manually using a 10 *u*l syringe (Hamilton) or by means of an autosampler, following the instructions for the particular model of instrument used. Injection port temperature is 250°C.

Carrier Gas: Hydrogen or helium, 99.999% pure. A moisture trap (Chrompak #7971 or equivalent) and an oxygen trap (Chrompak #7970 or equivalent) must be installed in the carrier line. An average linear velocity of 40 cm/sec for hydrogen and approximately 30 cm/sec for helium is used. Effects of temperature programming should be considered in setting this parameter.

Detector: The flame ionization detector should be designed for use with capillary columns. Hewlett-Packard instruments accomodate capillary coplumns by allowing for the addition of make-up gas (nitrogen) at the detector to compensate for the low flow rates of capillary columns. Some other manufacturers do not require make-up gas. If required, the combined flow rate (carrier and make-up)

should be approximately 30 ml/min at the detector. Hydrogen and air flows to the detector should be set according to instructions for the model of instrument used. Detector temperature is 270°C.

Column oven: Temperature programming is recommended. Operating temperatures will be dependent upon the liquid phase, length, and age of the column. The following is a typical program:

Initial temperature	170°C
Hold time	0
Rate	1 °/min
Final temperature	225°C
Final time	55 min

II. Establishing correction factors

For quantitation of fatty acid methyl esters, correction factors (CF) which compensate for differences in FID response to the fatty acids of interest, relative to the internal standard, need to be established for each fatty acid of interest.

From primary reference standards, prepare standard solutions at three concentrations, approximating the range of each fatty acid expected in 25 mg of sample. Carry all standards through the entire preparative and chromatography procedures outlined for samples above.

CALCULATION

I. Identification of Fatty Acid Methyl Esters

FAMEs from a refined menhaden oil (prepared by the above procedure) should be identified based on comparison of their equivalent chain length (ECL) values (4) with those of known standards in isothermal runs, by hydrogenation, by argentation TLC (5), and if available, by GC/MS. This oil should be stored for use as a secondary standard. Soft-gel encapsulated oil stored at 4°C is convenient because of its stability. For routine analysis, sample components may be tentatively identified by comparison of their relative retention times with those of the "standard" menhaden oil prepared and chromatographed at the same time as the samples.

II. Calculation of Correction Factors

For each fatty acid standard, calculate the correction factor (equation I). Since the plots of CF vs relative retention times for saturates, monounsaturates, and polyunsaturates give fairly smooth curves, correction factors can be predicted for fatty acids for which standards are not available. If the range of correction factors is narrow, the error introduced by this prediction is small. Typical correction factors are 1.02 for methyl arachidonate and 1.05 for methyl docosahexaenoate.

(Eqn I) $$K(t) = \frac{A(s) \cdot W(t)}{W(s) \cdot A(t)}$$

K(t) = correction factor for component t
A(s) = area of IS in reference chromatogram
W(s) = weight of IS added
A(t) = area of component t in reference chromatogram
W(t) = weight of component t in reference chromatogram

III. Calculation of Content of Each Fatty Acid Component by Weight

Using the correction factor for each fatty acid obtained above, equation II is used to calculate the content of each fatty acid methyl ester, by weight:

(Eqn II) $$mg/g\,\text{fatty acid} = \frac{K(t) \cdot A(t) \cdot W(s) \cdot 1000}{A(s) \cdot S \cdot 1.04}$$

K(t) = correction factor for component t
A(s) = area of IS in sample chromatogram
W(s) = weight of IS added, mg
A(t) = area of component t in sample chromatogram
S = weight of sample (oil), mg

Because of the cost, instability, and sometimes unavailability of polyunsaturated standard fatty acids, it is convenient to use a secondary standard for routine analyses. The composition of the "standard" menhaden oil is established by analyzing a minimum of 10 replicates of the oil at the same time as the primary standards and using the correction factors calculated from the available primary standards to determine composition. FAME are prepared from this secondary standard, in duplicate, with each group of samples analyzed. One replicate is chromatographed prior to the first sample and a second replicate after the last sample. If the results of the two replicates differ by more than 2% for EPA and DHA, the results of the entire set of samples should be rejected. If the average of the two replicates differs by more than 3% for EPA and DHA from the established composition of the oil, primary standards should be reanalyzed.

PRECISION

For duplicate analyses of menhaden oil FAME, the results for the major fatty acids should agree within ± 2%.

NOTES

1. Purity of commercial standards should be evaluated by GLC of the methyl esters prepared as described in this method and by quantitative TLC (Iatroscan TLC/FID) (6).

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2.3 FREE FATTY ACIDS

INTRODUCTION

The AOCS method (1) for determination of free fatty acids (FFA) in oil samples is the recommended method of the International Association of Fish Meal Manufacturers (IAFMM) (2). It has often proven unsatisfactory for the analysis of marine oils which may be yellow or red due to carotenoids, or brown due to oxidation products. In addition, the endpoint color of the phenolphthalein indicator may be masked by slight turbidity in marine oils or may exhibit endpoint fading, leading to difficulty in completing the titration accurately. Despite special precautions, such as multiple determinations, great difficulty may be experienced in obtaining consistent and accurate values for the FFA content of marine oil samples. Ke *et al*. (3) published an improved titrimetric method for the determination of FFA in fish oils, which has been modified by the BTM program for analysis using an autotitrator.

PRINCIPLE

In this method free fatty acids are titrated to endpoint with 0.05N NaOH. The method of Ke *et al*. (3) uses meta cresol purple (MCP) as the indicator and a mixed solvent system (chloroform:methanol:isopropanol) . MCP turns from yellow to purple at endpoint which avoids the problem of color interference from the marine oil. The mixed solvent system completely dissolves marine oil samples and mixes with at least 12 ml of aqueous NaOH titrant before becoming turbid. The Ke *et al*. method requires a significantly smaller sample size (1 g) than the AOCS method (up to 50 g) at low levels of free fatty acids. The titrant volume consumed by the titration is used to calculate the free fatty acid content as percent oleic acid. This method has been modified by the QA/QC Project for use with an autotitrator. In the automated titration, meta cresol purple is utilized in the procedure to visually monitor the reaction. The titrator, however, determines an endpoint in millivolts rather than the color endpoint (4). The titrant volume consumed is then converted by the instrument to percent oleic acid.

APPARATUS

* Autotitrator (Mettler DL-20 or equivalent)
* 5 ml burette (Mettler)
* Combination glass electrode (Mettler DG-112 or equivalent)
* Analytical balance, with RS232 interface (Mettler AE200 or equivalent)
* Printer-plotter (Epson FX-85 or equivalent)
* Disposable plastic titration beakers (Mettler)

* Disposable plastic transfer pipets
* 2 L volumetric flask
* 100 ml graduated cylinder

REAGENTS

* Chloroform: methanol:isopropanol (ACS grade), 2:1:2
* Meta cresol purple (MCP), 0.5% in distilled water (Fisher)
* NaOH, 0.05N (Fisher)
* Oleic acid, 99.5% pure (Nu-Chek Prep)
* Lithium chloride, reagent grade, 3M in methanol (electrode filling solution)
* Potassium biphthalate (store in dessicator)

PREPARATION OF STANDARDS & SAMPLES

1. Weigh 1 g oil (to the nearest 0.005 g) in a 100 ml plastic titration beaker.
2. Add 75 ml of solvent mixture and 4 drops of MCP indicator.

DETERMINATION

TABLE 2.3-1. AUTOTITRATOR CONFIGURATION FOR FREE FATTY ACIDS.

PARAMETER	CONFIGURATION	EXPLANATION
1	2	determination of all equivalence points
2	2	determination of steepest jump
3	0.01	predispensing amount (burette volumes)
4	0.3	maximum volume, in burette volumes
5	0	endpoint indicated in % FFA as oleic acid
6	30	stirring time, in seconds
7	11	representation of results: titration curve and initial signal
8	1	output to printer
9	0	series titration not used

1. Set the autotitrator to the configurations given in Table 2.2-1.
2. Calibrate the electrode with pH 4 and pH 7 buffers.

3. Determine concentration of the titrant solution (weekly): Switch instrument to concentration mode. Tare titration beaker on balance. Weigh out approximately 10 mg potassium biphthalate. Weight will automatically be entered on instrument. Add 75 ml H_2O. Press "run". Result should be approximately 0.25 meq/burette volume (= 0.05N * 5 ml burette volume). If below 0.225, discard titrant.
4. Determine solvent blank (daily): Switch instrument to blank mode. Add 75 ml solvent mixture to titration beaker plus 4 drops MCP. Run titration.
5. Determine sample: Tare titration beaker on balance. Add 1 g oil (to the nearest 0.005 g) to the beaker. Weight will be automatically entered into the instrument. Add 75 ml solvent mixture plus 4 drops MCP. Transfer titration beaker to instrument. Press "run" to initiate titration.

CALCULATION

The percentage of FFA is calculated by the instrument as oleic acid using the following formula:

$$result = C \cdot \frac{1}{weight} \cdot v \cdot conc$$

where,

v = titrant volume consumed up to the equivalence point in burette volume units

v * conc = raw result in milliequivalents

$$C, const = \frac{m}{10} \cdot z$$

where,

m = the molecular mass of oleic acid (g/mol)

z = valence of sample relative to titrant (eq/mol)

PRECISION

The standard deviation obtained by the manual method of Ke *et al.* for concentrations of FFA up to 13% is <1.5% of the FFA value. This is greatly reduced over that of the AOCS method, 11% of FFA value by different analysts and 6% of average FFA value by same analyst.

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2.4 ISOLATED TRANS FATTY ACIDS

INTRODUCTION

Both the AOCS and the AOAC have an official method for the determination of isolated *trans* isomers of fatty acids by infra red spectroscopy (IR); the AOAC uses the AOCS official method. The method utilized by the BTM Program for reporting isolated *trans* unsaturation is essentially the AOCS official method, modified to scan for low levels of *trans* unsaturation. In addition, individual *trans* fatty acids detected in the products are listed in the fatty acid profile obtained by the method described in section 2.2.

The AOCS method is applicable to the determination of isolated *trans* bonds in natural or processed long-chain acids, esters, and triacylglycerols which contain only small amounts (< 5%) of conjugated materials. The spectra of long-chain fatty acids exhibit a band of medium intensity at about 10.6 *u*, arising from a vibration of the carboxyl group. Correction for the contribution of this band to the 10.3 *u* band, along with correction for any "background" absorption at this wavelength, can be made by the baseline technique. However, if the isolated *trans* content of the long-chain fatty acid is sufficiently small, the correction will become the major factor in the measured absorption at 10.3 *u* and quantitative accuracy is not attainable. Therefore, long-chain fatty acids containing less than 15 % isolated *trans* isomers must be converted to their methyl esters before analysis by the infrared spectrophotometric method. Since the level of *trans* unsaturation found in the fish oil produced by the BTM Program is quite low, the oils are converted to methyl esters prior to analysis by IR. Ethyl ester products are analyzed without additional preparation.

This method is not applicable or is applicable only with specific precautions, to fats and oils containing large quantities (>5 %) of conjugated unsaturation, such as tung oil, or to materials containing functional groups which modify the intensity of the C-H deformation about the *trans* double bond, such as castor oil containing ricinoleic acid or its geometrical isomer ricinelaidic acid (12-hydroxy-*trans*-9-octadecenoic acid).

PRINCIPLE

In most naturally occurring vegetable fats and oils, unsaturated constituents contain only isolated, i.e., non-conjugated, double bonds in the *cis* configuration. These *cis* bonds may be isomerized to the *trans* configuration during extraction and processing procedures. Oxidation and partial hydrogenation promote isomerization from the naturally occurring *cis* to the *trans* isomers. Animal and marine fats may contain measurable amounts of naturally occurring *trans* isomers. Isolated *trans* bonds in long-chain fatty acids, esters and triacylglycerols are measured by infrared spectroscopy. An absorption band with maximum at about 10.3 *u*, arising from a C-H deformation about a *trans* double bond, is exhibited in the spectra of all compounds

containing an isolated *trans* group. This band is not observed in the spectra of the corresponding *cis* and saturated compounds. Measurement of the intensity of this absorption band under controlled conditions is the basis of a quantitative method for the determination of isolated *trans* content.

APPARATUS

* Infrared spectrophotometer, dual beam, covering the spectral region about 9 to 11 u, with wavelength scale readable to 0.01 *u*, and equipped with cell compartment holding 0.2 to 2.00 mm cells (Perkin Elmer 1420 or equivalent)
* Absorption cells, fixed thickness, 0.2 to 2.00 mm, NaCl or KBr windows. For use in null type instruments, pairs of cells matched to within 0.01 absorbance units are required. In split-beam type instruments, electronic balance of the two beams with both cells filled with CS_2 to within 0.01 absorbance units should be attained.
* Volumetric flasks, 5 ml and 10 ml volumes
* Centrifuge (Precision Centricone or equivalent) with head to acommodate 16x125 mm screw-capped vials and operate at a speed of at least 1300 rpm
* Hypodermic syringes with blunt needles for filling absorption cells
* Chart paper, calibrated in either transmittance or absorbance
* Analytical balance (Mettler AE200 or equivalent) capable of weighing $\pm$ 0.0002 g
* Nitrogen evaporator (Meyers N-Evap, or equivalent) with water bath capable of maintaining 37°C
* Disposable culture tubes, 16 x 125 mm, with teflon-lined screw caps (Kimble #45066a or equivalent)
* Temperature block modular heater, with block to support 16x125 mm tubes and controlled temperature at 100°C

REAGENTS

* Carbon disulfide, dry, ACS grade (see Note 2)
* Primary standards: methyl elaidate, methyl oleate, ethyl elaidate , and ethyl oleate, >99% pure (Nu-Chek Prep)
* Secondary standards: the secondary standards are calibrated against the primary standard and are used in order to conserve the primary standard. Other acids, esters and triglycerides may be used as long as they contain a defined proportion of *trans*-isomer as determined by calibration with the primary standard.
* Sodium sulfate, anhydrous (reagent grade)

* Isooctane (ACS reagent grade)
* Methanol, absolute (ACS reagent grade)
* Boron trifluoride (Supelco), 12% in methanol
* Sodium hydroxide (reagent grade), 0.5N in methanol
* Nitrogen, compressed, with in-line moisture trap
* NaCl (reagent grade), saturated aqueous solution

PREPARATION OF STANDARDS & SAMPLES

I. **Triacylglycerols**

1. Weigh approximately 0.15 g (to the nearest 0.0001g) into 16x125 mm culture tube.
2. Add 4 ml 0.5N methanolic NaOH. Cap tightly, vortex and heat in heater block at 100°C for 7 min.
3. Cool tube with tap water.
4. Add one 5 ml ampule BF_3, cap tightly, vortex and return to heater block for 5 min.
5. Cool tube to 37°C in water bath; add 2 ml isooctane and vortex.
6. Add 3 ml saturated NaCl and extract esters into isooctane by agitating the tube by hand approximately 1 min.
7. Centrifuge at 1300 rpm approximately 2 min.
8. With a Pasteur pipet, transfer the upper, isooctane layer to another tube containing anhydrous Na_2SO_4 (approximately 2 mm layer).
9. Cap, shake, allow to stand at least 20 min.
10. Filter (Whatman No. 1) the clear isooctane solution into a dry tube. Place the tube in a 37°C water bath; evaporate the solvent under a stream of dry N_2.
11. Add 1 ml carbon disulfide (stored over molecular sieves), mix thoroughly.

II. **Esters**

1. Weigh 0.15 g (to the nearest 0.0001 g) ester concentrate into a 16 x 125 mm culture tube.
2. Add 1 ml carbon disulfide and mix. Add anhydrous sodium sulfate, mix, allow to stand 20 min, filter (Whatman No. 1) into clean, dry tube.

III. Standards

1. Prepare mixed standards containing 5, 10, or 15% methyl elaidate in methyl oleate for the determination of triacylglycerols and 5,10,15% ethyl elaidate in ethyl oleate for determination of ethyl ester concentrates. The methyl elaidate/methyl oleate standards are prepared in the same manner as the triacylglycerol samples.
2. Weigh to the nearest 0.0001 g approximately 0.15 g of mixed standard into a 16 x 125 mm glass culture tube. Add 1 ml CS_2 and mix.

DETERMINATION

1. Fill matching absorption cells with CS_2, using a hypodermic syringe. With the cell in an upright position, inject the sample from the bottom allowing any trapped air bubbles to pass up through the cell. Place the cells in the reference and sample beam holders of the spectrophotometer. Scan from 150.0 to 60.0 *u* to check baseline and cleanliness of the cells.
2. Remove the CS_2 from the sample cell and dry the cell by gently pulling air through the cell with a syringe.
3. Fill the cell with mixed standard and place in sample beam holder. With the reference cell containing CS_2, scan from 150.0 to 60.0 *u*. Once a curve has been obtained for the required standard, it need not be repeated as long as the same instrument can be used with the same programming controls. However, if for any reason the exact programming cannot be duplicated when measuring a specific sample, the calibration curve for the standard must be determined. Routinely, at least the primary or reference samples are analyzed with each group of samples.
4. Flush the cell with CS_2, dry and fill with sample solution. Place the cell in the sample beam holder and scan.
5. If no measurable peak is observed at 10.3 *u*, the sample is reported as <5% *trans* unsaturation. If a measurable peak is observed, calculate the % *trans* unsaturation.

CALCULATION

1. On the chart, draw a baseline from 10.02 *u* to 10.59 *u* (Figure 2.3-1). Subtract the absorbance at the baseline (a) from the absorbance at 10.3 *u* maximum (b).

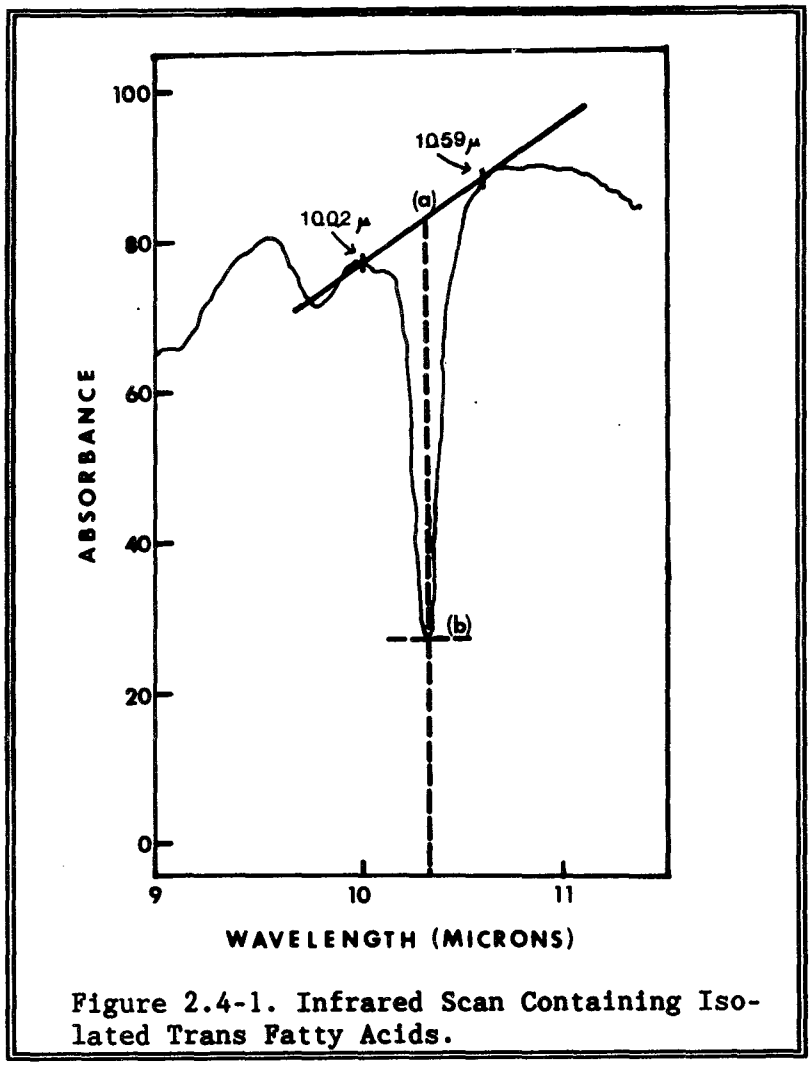

Figure 2.4-1. Infrared Scan Containing Isolated Trans Fatty Acids.

Percent *trans* fatty acids is calculated by the following equation:

$$\%TRANS = \frac{a(sample)}{a(standard)} x\,100$$

Where,

a = absorptivity = A/bc

and,

A = absorbance
b = internal cell length in cm
c = concentration of solution, g/l

REFERENCES

1. AOCS. (1980) ISOLATED *TRANS* ISOMERS, INFRARED ELECTROPHOTOMETRIC METHOD (Cd 14-61, REAPPROVED 1973), *in* OFFICIAL AND TENTATIVE METHODS, THIRD EDITION, R.O. WALKER (ED.). AMER. OIL CHEM. SOC., CHAMPAIGN, IL, 5p.

2. AOAC. (1984) ISOLATED *TRANS* ISOMERS IN MARGARINES AND SHORTENINGS, INFRARED SPECTROSCOPIC METHOD (#28.086-28.091, FINAL ACTION), *in* OFFICIAL METHODS OF ANALYSIS OF AOAC, FOURTEENTH EDITION, S. WILLIAMS (ED.). ASSOC. OFC. ANAL. CHEM. ARLINGTON, VA, pp. 518-519.

3. FIRESTONE, D., P. LABOULIERE. (1965) DETERMINATION OF ISOLATED *TRANS* ISOMERS BY INFRARED SPECTROPHOTOMETRY. JAOAC 48:437-443.

2.5 UNSATURATED FATTY ACIDS (IV)

INTRODUCTION

The iodine value is a measure of the unsaturation of fats and oils. The AOCS has an official method (1,2) for the determination of the iodine value, the Wijs Method (Cd 1-25). The AOCS method utilizes titration with sodium thiosulfate to a color endpoint. The method has been modified for use with autotitrators and commercially prepared Wijs solution. The autotitrator utilizes a voltametric endpoint. This automated method is used by the BTM Program.

PRINCIPLE

An oil sample is incubated for 1 hr in the dark with an excess of iodine in acetic acid (Wijs solution). The amount of iodine absorbed is proportional to the number of double bonds present in the oil and is determined by a back-titration with sodium thiosulfate. The iodine value is expressed as grams of iodine absorbed per 100 grams of sample. The method is applicable to all normal fats and oils not containing conjugated systems.

APPARATUS

* Autotitrator (Mettler DL-20 or equivalent)
* Mettler DM140 electrode, or equivalent capable of determining $\pm$0.05 pH units
* 20 ml burette (Mettler)
* Printer (Epson FX-85 or equivalent)
* 250 ml glass titration flasks (Mettler ME-23515 or equivalent)
* Analytical balance, capable of weighing to $\pm$0.0001g, with RS232 interface (Mettler AE200 or equivalent)
* Automatic pipet (Rainin Pipetman or equivalent)

REAGENTS

* Potassium iodide (ACS grade), 150 g/l in distilled H_2O. The H_2O should be de-gassed by boiling and then cooling to room temperature before making the solution.
* Chloroform (ACS grade)
* Wijs iodine solution (Fisher)
* Sodium thiosulfate ($Na_2S_2O_3*5H_2O$), ACS grade, 0.1N in distilled H_2O.
* KIO_3, 1 N standard solution (Fisher)

PREPARATION OF STANDARDS AND SAMPLES

1. Prepare fish oil and ester concentrate samples for analysis by assuring that no particulate matter or moisture is present; filtration may be performed.
2. The weight of the sample must be such that there will be an excess of Wijs solution of 50-60% of the amount added, i.e. 100-150% of the amount absorbed. Table 2.4-1 provides a guide to the sample weight required for determination at different iodine values:

TABLE 2.5-1. SAMPLE WEIGHTS REQUIRED FOR IODINE VALUE DETERMINATION.

EXPECTED IODINE VALUE	SAMPLE WEIGHT g		WEIGHING ACCURACY ± g
	100% EXCESS	150% EXCESS	
<3	10.000	10.000	0.001
3	10.576	8.4613	0.005
5	6.3460	5.0770	0.0005
10	3.1730	2.5384	0.0002
20	1.5865	0.8461	0.0002
40	0.7935	0.6346	0.0002
60	0.5288	0.4231	0.0002
80	0.3966	0.3173	0.0001
100	0.3173	0.2538	0.0001
120	0.2644	0.2115	0.0001
140	0.2266	0.1813	0.0001
160	0.1983	0.1587	0.0001
180	0.1762	0.1410	0.0001
200	0.1586	0.1269	0.0001

DETERMINATION

1. Set autotitrator with the configurations shown in Table 2.4-2.
2. Determine concentration of the titrant (weekly): Switch the instrument to concentration mode. Set parameter 4 at 0.3 and parameter 6 at 75. Pipet 5 ml KIO_3 solution into titration beaker. Add 20 ml chloroform and 25 ml Wijs solution. Place the beaker on the titrator. Turn on the stirrer. Press "run". Result should be 2.0 meq/burette vol. (= 0.1N * 20 ml burette vol.)
3. Weigh approximately 0.1 g of fish oil accurately (to the nearest 0.0001 g) into a 250 ml titration flask. Add 20 ml of chloroform.

4. Pipet 25 ml of the Wijs solution into the titration flask containing the sample and turn on the stirrer to ensure good mixing.
5. Prepare and conduct at least 2 blank determinations with each group of samples.
6. Store the flasks in a dark place for 1 hr at 25 ± 5°C.
7. Remove the flasks from storage and add 20 ml of KI solution, followed by 100 ml of distilled water.
8. Configure the DL-20 autotitrator with the settings listed in Table 2.4-2.

TABLE 2.5-2. AUTOTITRATOR CONFIGURATION FOR IODINE VALUE.

Parameter No.	Value	Definition
1	2	titration mode: steepest jump
2	1	reaction type: standard titration
3	0	predispensing: none
4	3(sample);2(blank)	maximum volume in burette volumes
5	0	endpoint indicated in mV
6	15	stirring time in seconds
7	11	output format: titration curve and initial signal
8	1	output to printer

9. Calibrate the electrode using standard buffer solutions.
10. Enter 12.69 for constant, 2 for "const reagent".
11. Place titration flask on the titration head, enter sample weight, press run.

CALCULATION

In the AOCS standard method, the iodine value is calculated as follows:

$$IV = (B - S) \cdot N \cdot \frac{C}{weight\,of\,sample}$$

where,

$$C = -\frac{m}{10 \cdot Z} = 12.69$$

N = normality of titrant

B = volume of titrant used for blank determination

S = volume of titrant used for sample determination

M = molecular weight of the titrant

Z = valence

The autotitrator reports the iodine value in % iodine absorbed. The blank is subtracted automatically. The constant of 12.69 and the sample weight are entered in steps 8 and 9 of the "Determination".

PRECISION

For samples analyzed in triplicate, the relative standard deviation is routinely less than 2%.

REFERENCES

1. AOCS. (1974) IODINE VALUE (Cd 1-25, REAPPROVED 1973), *in* OFFICIAL AND TENTATIVE METHODS, THIRD EDITION, R.O. WALKER (ED.). AMER. OIL CHEM. SOC., CHAMPAIGN, IL, 5p.

2. IAFMM. (1981) RECOMMENDED METHOD OF ANALYSIS FOR DETERMINATION OF IODINE VALUE OF FISH OILS (WIJS METHOD). FISH OIL BULL. No. 4, JUNE, 4p.

3. METTLER. (1983) IODINE VALUE OF OILS AND FATS ACCORDING TO WIJS, *in* METTLER APPLIC. BULL. No. 162, 1p.

2.6 DEUTERATED FATTY ACIDS

INTRODUCTION

Long chained highly unsaturated vinyl deuterated fatty acids have been virtually unavailable to investigators interested in n-3 fatty acid research. Because of their lack of availability, standard methodology has not evolved for the QA of these compounds. Therefore a combination of analytical methods applicable to fatty acids along with standard chemical investigation for structure and molecular integrity are applied to the QA of such vinyl deuterated fatty acids as linolenic acid (18:3n-3, d6), arachidonic acid (20:4n-6, d8), eicosapentaenoic acid (20:5n-3, d10), docosahexaenoic acid (22:6n-3, d12).

PRINCIPLE

The quality assurance of vinyl deuterated n-3 fatty acids is accomplished utilizing a variety of analytical methodologies. The product is tested by TLC for presence of free fatty acids, fatty acid esters and other components. If TLC indicates that the product is relatively pure, then the sample is analyzed for fatty acid composition utilizing capillary GC. If the elution pattern is consistent with the presence of a single component (>98%) and the elution time is identical with an authentic standard for the compound then the product is subjected to GC/MS, NMR and IR: the mass is determined (GC/MS), the deuterated structure is verified (NMR) and the percent *trans* product (IR) is determined (<5%).

DETERMINATION

1. Conduct semi-quantitative TLC (1) to determine the number of major components present; if only one component is present as expected, verify that it is a free fatty acid.
2. Conduct TLC/FID to determine the amount of free fatty acid in the material (2).
3. Conduct preparative TLC to isolate the free fatty acid band for GC studies (3).
4. Prepare the fatty acid methyl ester (FAME) derivatives (BF_3) of the isolated free fatty acid band and analyze by GC (4). Identify and quantify all major and minor fatty acids present. Examine the data for shifts in relative retention time, for the specific FAME under study, from that of the non-deuterated fatty acid as an indication of deuteration.
5. Analyze the FAME by GC/MS (5) to verify the molecular weight as being compatible with that of the FAME if it were fully vinyl-deuterated. Compare the spectrum for the FAME under study with that for an authentic standard of the non-deuterated FAME to

verify the fragmentation pattern. If a molecular ion is present, determine the molecular weight. Verify that <0.001% d_o is present.

6. Analyze the FAME by NMR to verify the structure and the distribution of deuterium. Compare the NMR splitting pattern and chemical shifts with that of an authentic standard of non-deuterated FAME.

REFERENCES

1. Refer to Section 2.1 of this manual.
2. ACKMAN, R.G. (1981) FLAME IONIZATION DETECTION APPLIED TO THIN LAYER CHROMATOGRAPHY ON COATED QUARTZ RODS. METH. ENZYMOL. 72:205-252.
3. KATES, M. (1975) TECHNIQUES OF LIPIDOLOGY. *in*, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, T.S. WORK AND E. WORK (EDS.), AMERICAN ELSEVIER PUBLISHING CO., NEW YORK, pp. 269-610.
4. Refer to Section 2.2 of this manual.
5. Support is provided by NIH/ADAMHA by providing verification of GC/MS studies.

3 CHOLESTEROL

INTRODUCTION

Marine oils contain significant concentrations of cholesterol that are removed or greatly reduced during processing of the BTM oils and esters. Concentrations of cholesterol are measured at various stages during production of the test materials to monitor processing techniques, and in the final products to assure quality.

An official method for the gas chromatographic determination of cholesterol has received final action by the AOAC (1). This method requires isolation of the sterols by TLC prior to analysis by GC using a packed column. A simplified method for determination of cholesterol and some plant sterols in fishery-based products is described by Kovacs *et al.* (2). The method utilized by the BTM Program is a modification of methodology developed at the USDA Human Nutrition Research Center in Beltsville, MD (3) that permits the determination of cholesterol, major plant sterols and alpha- and gamma-tocopherols from a single analysis by capillary gas chromatography (also see Section 5.3.2).

PRINCIPLE

A sample containing approximately 0.1 g of lipid (fat, oil or lipid extract) is mixed with known weight of the internal standard (5-*a*-cholestane) and saponified at 80°C with a known weight of aqueous KOH in the presence of pyrogallol. The unsaponifiables are extracted with cyclohexane, the solvent is removed under nitrogen, and the trimethylsilyl ethers are prepared using N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane in pyridine. This derivatized total unsaponifiable fraction is chromatographed on a non-polar fused silica wall-coated open-tubular (WCOT) column. Cholesterol is identified by comparison of its peak retention time with that of an internal standard and with external cholesterol standards prepared and chromatographed in an manner identical to the samples. Linear regression equations derived from analysis of the external standards are used to calculate the amount of cholesterol in the samples.

APPARATUS

* Gas chromatograph (HP5840 or equivalent), equipped for use with capillary columns (0.22-0.32 mm id), with an FID detector and optional autosampler
* Non-polar flexible fused-silica capillary column, 25m x 0.2mm x 0.3*u* film thickness (Hewlett-Packard, Ultra 2) or 50m x 0.22mm x 0.25*u* film thickness (J&W Scientific, DB-1)
* Centrifuge (Centra 4 International Centrifuge or equivalent) with rotor to accomodate 25 x 150 mm screw capped tubes, capable of operating at 1300 rpm

* Nitrogen evaporator with water bath capable of 37°C (Meyers N-Evap or equivalent)
* Water bath, 80°C (Precision Scientific or equivalent)
* Vortex mixer
* Pasteur pipettes, disposable
* Automatic positive displacement pipet capable of delivering 1ml ± 0.01ml (Eppendorf Repeater Pipet Model No. 2226 000-6 or equivalent)
* Centrifuge tubes, 25 x 125mm, with teflon lined screw caps
* Disposable culture tubes, 16 x 125 mm, with teflon-lined screw caps, silanized
* Analytical balance (Mettler AE163 or equivalent)
* Mechanical sample mixer (Fisher-Kendall or equivalent)
* Hamilton syringe, 10 *u*l (#1750 or equivalent)
* Moisture trap (Chrompak #7971 or equivalent)
* Oxygen trap (Chrompak #7970 or equivalent)

REAGENTS

* Hexane, ACS reagent grade
* Isooctane, ACS reagent grade
* Butylated hydroxytoluene (BHT), reagent grade
* Nitrogen, compressed
* KOH (reagent grade), aqueous saturated solution (prepare fresh daily)
* Pyridine, silation grade (Pierce Chemical Co.)
* N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (Supelco Sylon BFT or equivalent)
* Sylon CT (Supelco)
* Cholesterol standard (Eastman Kodak)
* 5-*a*-Cholestane (Supelco)
* Absolute ethanol, reagent grade, degassed (Note 2)
* Pyrogallol, reagent grade (Malinckrodt), 3% w/v in degassed absolute ethanol
* Helium or hydrogen (99% pure or better), carrier gas
* Nitrogen, compressed, make-up gas
* Cyclohexane, degassed (Note 2)

PREPARATION OF STANDARDS

1. **Internal Standard:** 40-50 *ug*/ml 5-*a*-cholestane + 20 *ug*/ml BHT in isooctane.

2. **Reference Standard:** Prepare a stock reference solution, 500 *ug*/ml cholesterol in isooctane containing 1% pyridine (v/v). Appropriate dilutions are made for each set of samples to establish a calibration curve (50-500 *ug*) using at least three concentrations.

PREPARATION OF SAMPLES

1. Weigh approximately 100 mg (to the nearest 0.0001 g) of oil or ester concentrate into a 50 ml screw cap centrifuge tube.
2. Add 1.0 ml internal standard to each sample and cholesterol standard.
3. Evaporate the solvent using a stream of nitrogen and a 37°C water bath.
4. Add 8 ml pyrogallol-ethanol solution and 0.5 ml saturated KOH.
5. Cap tubes tightly and vortex 5 sec.
6. Heat tubes 8 min in 80°C water bath; shake vigorously after 1,2 and 4 min so that the solution washes tube sides and caps.
7. Remove tubes and cool in cold water for 15 sec.
8. Add 10 ml cyclohexane and vortex for 5 sec.
9. Add 6 ml distilled water and shake for 15 min (mechanical mixer).
10. Centrifuge for 15 min (1300 x g).
11. Transfer cyclohexane layer to a 16 x 125 mm culture tube.
12. Evaporate the cyclohexane with a gentle stream of nitrogen with the culture tube in a 37°C water bath.
13. Add 100 *u*l pyridine and 100 *u*l Sylon BFT; vortex 5 sec and let stand 15 min at room temperature. The sample is now ready for analysis by GC.

DETERMINATION

The extracted samples are chromatographed isothermally on a fused silica capillary column (DB-1 or HP Ultra 2) using an all-glass split injection port with a split ratio of approximately 1:40. Carrier gas is helium (99.99% pure) with in-line moisture and oxygen traps. The average linear velocity is approximately 30 cm/sec. In instruments requiring make-up gas when using capillary columns, nitrogen is used as the make-up gas to the flame ionization detector (FID). The combined flow rate at the detector (carrier + makeup) is 30 ml/min. The following temperature parameters are used on the HP5840 GC: injection port - 300°C; Detector - 300°C; column oven - 280°C.

A 1 *u*l sample is injected either manually using a 10 *u*l syringe or by autosampler (Note 3).

CALCULATION

A relative retention time is calculated for the cholesterol standard versus that of the internal standard. The cholesterol peak is then identified in the sample by comparison of the relative retention time with that of the cholesterol standard.

A calibration curve is created by analyzing at least three concentrations of cholesterol standard, where x = the weight ratio and y = peak area ratio of the cholesterol standard to the internal standard. The slope and the y-intercept are determined by linear regression. The cholesterol peak in the sample is quantitated using the formula:

$$mg/g = \left(\frac{A(t)}{A(i)} - a\right) \cdot \frac{W(i)}{m \cdot W(s)}$$

W(i) = weight of internal standard, mg
A(i) = area of internal standard
A(t) = area of component T
W(s) = sample weight, g
m = slope
a = y-intercept

PRECISION

The average standard deviation for this assay is $\pm$ 2% for duplicate analyses.

NOTES

1. The purity of cholesterol standards should be evaluated by TLC and GC (sections 2.1 and 2.2 of this manual).
2. Degassing solvents:
   a. Water - boil 15 min. Cool. Stopper tightly and store at room temperature.
   b. Cyclohexane and absolute ethanol - degas under vacuum using a rotary evaporator.
3. Sylon BFT is corrosive and will attack syringe needles and plungers, thus they should be rinsed promptly. Pryidine will cause some elastomers to swell (e.g., vitons). Glass and teflon are resistant to pyridine. TMS ethers are unstable if stored in BSTFA. To preserve the derivative, add 1 ml petroleum ether and remove solvent and reagent with a gentle stream of nitrogen. Dissolve residue in isooctane.

REFERENCES

1. AOAC. (1984) FATS (ANIMAL) IN VEGETABLE FATS AND OILS (DETERMINATION OF CHOLESTEROL), GAS CHROMATOGRAPHIC METHOD, *in* OFFICIAL METHODS OF ANALYSIS OF AOAC, FOURTEENTH EDITION, S. WILLIAMS (ED.) ASSOC. OFC. ANAL. CHEM. ARLINGTON, VA, (#28.110-28.117, FINAL ACTION), pp. 522-523.

2. KOVACS, M.I.P, W.E. ANDERSON, R.G. ACKMAN. (1979) A SIMPLE METHOD FOR THE DETERMINATION OF CHOLESTEROL AND SOME PLANT STEROLS IN FISHERY-BASED PRODUCTS. J. FD. SCI. 44:1299-1305.

3. SLOVER, H.T., R.H. THOMPSON, JR, AND G.V. MEROLA. (1983) DETERMINATION OF TOCOPHEROLS AND STEROLS BY CAPILLARY GAS CHROMATOGRAPHY. J. AMER. OIL CHEM. SOC. 60:1524-1528.

4 FATTY ACID OXIDATION PRODUCTS

4.1 PEROXIDES (PV)

INTRODUCTION

Peroxide value is used as a primary measurement of oxidation in oils. An official method exists in both the AOCS (1) and the AOAC (2) for the determination of the peroxide value. The AOCS method is used with an autotitrator by the BTM Program.

PRINCIPLE

This titrimetric method determines all substances which oxidize potassium iodide, in terms of milliequivalents of peroxide per 1000 grams of sample. These substances are assumed to be peroxides or other similar products of fat oxidation. The method is applicable to all normal fats and oils including margarine and is highly empirical. Any variation in procedure may result in variation in results.

APPARATUS

* Autotitrator, Mettler DL20 or equivalent
* Analytical balance, capable of measuring $\pm$0.001 g, with RS232 interface
* Printer, Epson FX-85 or equivalent
* Electrode (Mettler DM140)
* 5 ml burette (Mettler)
* 100 ml disposable titration beakers
* Pipetman P1000 pipet and tips (or equivalent)
* Graduated cylinder, 50 ml
* Timer, 60 minute

REAGENTS

* Glacial acetic acid:chloroform, 3:2 (v:v) (reagent grade)
* Sodium thiosulfate, 0.05N (0.1N reagent diluted with distilled water)
* Potassium iodide (Fisher ACS grade), 30 g/21 ml boiled (degassed) distilled H_2O (Note 1)
* Starch indicator solution: 1.0% of soluble starch in distilled water.
* KIO_3, 0.1 N standard solution (Fisher)

DETERMINATION

TABLE 4.1-1. AUTOTITRATOR CONFIGURATIONS FOR PEROXIDE VALUE.

Parameter No.	Value	Description
1	2	Titration mode: equivalence point.
2	1	Reaction type: standard titration.
3	0	No predispensing.
4	0.3	Maximum volume in burette volumes.
5	0	mV endpoint.
6	75	Stirring time.
7	11	Report: titration curve, initial signal. Output to printer.
8	1	

1. Configure the autotitrator with the settings shown in Table 4.4-1.
2. Calibrate the electrode using standard buffer solutions.
3. Determine concentration of the titrant (weekly): Switch the instrument to concentration mode. Pipet 0.5 ml KIO_3 into titration beaker. Add 30 ml acetic acid:chloroform solution. Add 0.5 ml KI solution. Allow solution to stand with occasional swirling exactly 1 min. Place the beaker on the titrator. Turn on the stirrer. Press "run". Result should be 0.25 meq/burette vol. (= 0.05N * 5 ml burette vol.).
3. Enter 1000 for "const", 20 for "const reag", 0 for "blank".
4. Tare titration beaker on the balance. Weigh approximately 5 g ($\pm$0.05 g) oil into titration beaker. Press "run" to enter the weight on the autotitrator.
5. Add 30 ml acetic acid:chloroform solution. Pipet in 0.5 ml KI solution.
6. Allow the solution to stand, with occasional shaking, for exactly 1 minute and then add 30 ml of distilled water. A timer may be used.
7. Immediately add 30 ml distilled H_2O and 5 ml starch indicator solution.
8. Place titration beaker on the titration head. Press "run".

CALCULATION

The autotitrator automatically calculates the peroxide value as milliequivalents of peroxide per 1000 g of sample, using the following formula:

$$PV = \frac{(S - B) \cdot N \cdot 1000}{\text{weight of sample}}$$

where, B = titration of blank
S = titration of sample
N = normality of sodium thiosulfate solution

PRECISION

For samples analyzed in triplicate the relative standard deviation is generally less than 5%.

NOTES

1. Store saturated KI solution in the dark. Determine the concentration of the KI solution daily by adding 2 drops of starch solution to 0.5 ml of KI solution in 30 ml acetic acid:chloroform. If a blue color is formed which requires more than 1 drop of 0.1 N sodium thiosulfate solution to discharge, discard the iodide solution and prepare a fresh solution.

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4.2 ALDEHYDES (AV)

INTRODUCTION

The p-anisidine value is a standard IUPAC method for the measurement of aldehydes (principally 2-alkenals) present as oxidation products in the oils and fats (1). It is applicable to animal and vegetable oils and fats. The standard IUPAC method is used by the BTM Program for the analysis of aldehydes in fish oil and esters of fish oil.

PRINCIPLE

P-anisidine reacts with aldehydic compounds in oils and fats in the presence of acetic acid to produce a yellowish product. The intensity of color of the product is measured by absorbance at 350 nm. The intensity depends not only on the amount of aldehydes present, but also on their structure. It has been found that a double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance four or five times. Thus the 2-alkenals contribute substantially to the value. The reaction between p-anisidine and aldehydes involves the formation of water. Hence, the presence of moisture in any of the reagents or in the sample leads to incomplete reaction and, consequently, low values. The p-anisidine value is defined by convention as 100 times the optical density, measured in a 1 cm cell, of a solution containing 1 g of the oil in 100 ml of a mixture of solvent and reagent.

APPARATUS

* 10-ml test tubes, with teflon-lined screw caps
* 25-ml volumetric flasks.
* Automatic positive displacement pipet (1 ml capacity)
* Automatic positive displacement pipet (5 ml capacity)
* Spectrophotometer capable of measuring absorbance at 350 nm.
* Glass spectrophotometer cuvettes, paired 1 cm
* Analytical balance, capable of weighing to 0.001 g
* Timer, 60 minute

REAGENTS

* Isooctane (ACS reagent grade)
* Glacial acetic acid, analytical reagent quality (Note 1)
* p-Anisidine (Sigma), 2.5 g/L in glacial acetic acid (Note 2)

PREPARATION OF SAMPLES

1. The sample should be clear and dry. Particulate matter may be removed from the sample by filtration.

DETERMINATION

1. Weigh 0.5 - 4.0 g (to the nearest 0.001 g) oil or ester sample into a 25 ml volumetric flask. Dissolve and dilute to volume with isooctane.
2. Measure absorbance (A_b) of the sample solution at 350 nm with the reference cell filled with isooctane.
3. Pipette 5.0 ml of the oil solution into a test tube. Pipette 5.0 ml of isooctane into a second test tube (solvent blank). Add 1.0 ml of the p-anisidine solution to each tube. Shake.
4. Incubate the tubes at room temperature exactly 10 minutes. Measure the absorbance (A_s) of the solution in the sample tube at 350 nm, using the solvent blank in the reference cell.

CALCULATION

The anisidine value (A.V.) is given by the formula:

$$A.V. = 25 \cdot \frac{1.2(As - Ab)}{m}$$

where,

A_s = the absorbance of the oil solution after reaction with the p-anisidine reagent;

A_b = the absorbance of the solution of the oil;

m = the mass, in g, of the sample.

PRECISION

Duplicate analyses generally display less than 5% relative standard deviation.

NOTES

1. Since glacial acetic acid is highly hygroscopic, it is essential to check its moisture content by a Karl Fischer determination (section 7 in this manual). If the content exceeds 0.1 percent, the acetic acid must be discarded.
2. P-anisidine solutions having an absorbance greater than 0.200 when measured in a 1 cm cell at 350 nm against isooctane as a blank should be discarded.

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4.3 POLAR OXIDATION PRODUCTS

INTRODUCTION

A method for the determination of polymers and polar oxidation products has received final action by the AOAC (1). An internal standard (triheptadecanoin) is added to the oil prior to preparation of the fatty acid methyl esters (FAME). The esters are analyzed by packed column gas chromatography and the percent of polymers is determined as the calculated percent of non-eluting material (NEM), based on the sum of the uncorrected areas for the individual FAME. It has been suggested that for the determination of marine oils or animal fats, a different internal standard should be used or a second ester should be prepared for analysis without internal standard, and correction must be made for the area of minor components that may coelute with the internal standard (2). The method utilized by BTM Program for the determination of non-eluting materials associated with heating and storage of oils (polymers and polar oxidation products) is a refinement of the AOAC method, utilizing capillary gas chromatography and corrected peak areas.

For the analysis of marine lipids, capillary columns offer distinct advantages over packed columns. Most geometric and positional isomers commonly found in the C-12 to C-24 range are separated in 30-40 min. Background noise is also reduced allowing detection of minor fatty acids.

Although little is gained in accuracy by application of correction factors to GC peak areas measured manually, correction factors can enhance the accuracy of electronically integrated analyses. The advantage is more significant for the analysis of marine oils than vegetable oils because of the wide range of fatty acids and, therefore, the wider range of correction factors encountered.

PRINCIPLE

An accurately weighed sample of oil is mixed with a known amount of internal standard, saponified with methanolic NaOH, and reacted with boron trifluoride in methanol to produce fatty acid methyl esters. The esters are extracted into isooctane and analyzed by GC with flame ionization detection. Separation is achieved on a polar fused silica capillary column and peak areas are measured by electronic integration.

Fatty acid standards are used for identification of the FAME and determination of correction factors. The sum of the weights (g/100 g oil) of the individual FAME is used to calculate the percent triacylglycerol in the oil. The percent of non-eluting material is then determined by difference.

APPARATUS

* Gas chromatograph (Hewlett Packard 5890 or equivalent), designed to accept capillary columns (0.22-0.32 mm ID), with an FID detector and an electronic integrator
* Polar flexible fused silica capillary column (0.22-0.32 mm ID) at least 30 m in length, DB225 (J and W Scientific) or Supelcowax -10-polyethylene glycol (Supelco)
* Centrifuge (Precision Centricone or equivalent) with rotor to accomodate 16x125 mm screw cap culture tubes and capable of at least 1300 rpm
* Nitrogen evaporator with water bath capable of maintaining 37°C (Meyers N-Evap or equivalent)
* Vortex mixer
* Pasteur pipettes, disposable
* Automatic positive displacement pipettor capable of delivering 1 ml $\pm$ 0.01 ml (Eppendorf repeater pipette No. 2226 000-6 or equivalent)
* Disposable culture tubes, 16x125 mm, with teflon lined-screw caps (Kimble #45066-A or equivalent)
* Temperature block modular heater, with block to accomodate 16x125 mm tubes and controlled temperature at 100 $\pm$ 2°C
* Hamilton syringe, 10 *ul*
* Moisture trap (Chrompak #7971 or equivalent)
* Oxygen trap (Chrompak #7970 or equivalent)

REAGENTS

* Isooctane (ACS reagent grade)
* Methanol, absolute (ACS reagent grade)
* Boron trifluoride, 12% in methanol (Supelco)
* Sodium hydroxide (reagent grade), 0.5N in methanol
* Sodium chloride (reagent grade), saturated aqueous solution
* Butylated hydroxytoluene (BHT), reagent grade (Kodak)
* Methyl tricosanoate, 99+% (Nu-chek Prep)
* Simple triacylglycerol standards for the major fatty acids to be determined. Methyl esters may be used for fatty acids for which triacylglycerol standards are not available (Nu-chek Prep, Supelco). (Note 1)
* Helium or hydrogen (99% pure or better), carrier gas
* Nitrogen, compressed, dried (for solvent removal and make-up gas)

PREPARATION OF STANDARDS AND SAMPLES

I. **Standards**

1. **Internal Standard.** Weigh approximately 100mg (to the nearest 0.1 mg) methyl tricosanoate into a 100 ml volumetric flask and make to volume with isooctane containing 20 *u*g/ml BHT.
2. **Reference Standards.** Weigh to nearest 0.1 mg into a volumetric flask and make to volume with isooctane. Cost and availability of the standards must be considered in determining weights and volumes used.

II. **Preparation of Esters**

1. Weigh approximately 25 mg (to the nearest 0.0001 g) into a 16x125 mm screw-capped culture tube.
2. Add 1.0 ml methyl tricosanoate internal standard (IS). Mix thoroughly and evaporate to dryness under a stream of N_2 (in N-Evap).
2. Add 1 ml of 0.5N NaOH, cap tightly, vortex, and heat for 5 min at 100°C in heating block.
3. Cool with tap water.
4. Add one ampule (2 ml) boron trifluoride; cap tightly, vortex, and return the tube to the heat block for 5 min.
5. Cool to 37°C in water bath, add 1 ml isooctane and vortex.
6. Add 3 ml saturated NaCl and extract into isooctane by agitating tube for approximately 1 min.
7. Centrifuge at 1300 rpm for approximately 2 min.
8. With a pasteur pipet, transfer the (upper) isooctane phase to a small vial containing anhydrous Na_2SO_4 (approximately a 2 mm layer).
9. Cap, shake, and allow to stand for approximately 20 min.
10. If autosampling is used, transfer the clear isooctane (containing methyl esters) to 1 ml autosampler vial and seal with a crimp cap. If manual injection is used, transfer the isooctane to a vial with a teflon lined crimp or screw cap.

DETERMINATION

One *u*l of sample is injected manually using a 10 *u*l syringe (Hamilton) or by means of an autosampler, following the instructions for the particular model of instrument used.

I. **Gas Chromatography System**

Injection System: The injection port should be fitted with an all-glass, split-injection line. The split ratio used is approximately 1:100. Injection port temperature is 250°C.

Carrier Gas: Hydrogen or helium, 99.99% puritry. A moisture trap (Chrompak #7971 or equivalent) and an oxygen trap (Chrompak #7970 or equivalent) must be installed in the carrier gas line. The average linear velocity of 40 cm/sec for hydrogen and approximately 30 cm/sec for helium is used. Effects of temperature programming should be considered in setting this parameter.

Detector: The flame ionization detector should be designed for use with capillary columns, allowing addition of make-up gas (nitrogen) at the detector to compensate for the low flow rates of capillary columns. The combined flow rate (carrier and make-up) should be approximately 30 ml/min at the detector. Hydrogen and air flows to the detector should be set according to instructions for the model of instrument used. Detector temperature is 270°C.

Column oven: Temperature programming is recommended. Operating temperatures will be dependent on the liquid phase, length, and age of the column. The following is a typical program:

Initial temperature	170°C
Hold time	0
Rate	1 °/min
Final temperature	225°C
Final time	55 min

II. Establishing Correction Factors

For quantitation of fatty acid methyl esters, correction factors (CF) which compensate for differences in FID response to the fatty acids of interest, relative to the internal standard, need to be established for each fatty acid of interest.

From primary reference standards, prepare standard solutions for three concentrations, approximating the range of fatty acid concentrations expected in 25 mg of sample. Chromatograph as outlined above.

CALCULATION

1. Identification of Fatty Acid Methyl Esters

FAMEs from a refined menhaden oil (prepared by the above procedure) should be identified based on comparison of their equivalent chain length (ECL) values (4) with those of known standards in isothermal runs, by hydrogenation, by argentation TLC (5), and if available, by GC/MS. This oil should be stored for use as a secondary standard. Soft-gel encapsulated oil stored at 4°C is convenient because of its long-term stability. For routine analysis, FAME samples may be tentatively identified by comparison of their relative retention times with those of "standard" menhaden oil prepared and chromatographed at the same time as the samples.

2. Calculation of Correction Factors

For each fatty acid standard, calculate the correction factor (Equation I). Since the plots of CF vs relative retention times for saturates, monounsaturates, and polyunsaturates give fairly smooth curves, correction factors can be predicted for fatty acids for which standards are not available. If the range of correction factors is narrow, the error introduced by this prediction is small. Typical correction factors are 1.02 for methyl arachidonate and 1.05 for methyl docosahexanoate.

(Eqn I) $$K(t) = \frac{A(s) \cdot W(t)}{W(s) \cdot A(t)}$$

K(t) = correction factor for component t
A(s) = area of IS in reference chromatogram
W(s) = weight of IS added
A(t) = area of component t in reference chromatogram
W(t) = weight of component t in reference chromatogram

3. Calculation of content of each fatty acid component by weight

Using the correction factor for each fatty acid obtained above, Equation II is used to calculate the content of each fatty acid methyl ester, by weight:

(Eqn II) $$T = \frac{K(t) \cdot A(t) \cdot W(s) \cdot 1000}{A(s) \cdot S}$$

T = amount of FAME t in mg/100 mg
K(t) = correction factor for component t
A(s) = area of IS in sample chromatogram
W(s) = weight of IS added, mg
A(t) = area of component t in sample chromatogram
S = weight of sample (oil), mg

(Eqn III) $$\%NEM = 100 - sumT$$

PRECISION

For duplicate analyses, the results should agree to within $\pm$ 5%.

NOTES

1. Because of the cost, instability, and sometimes unavailability of standard polyunsaturated fatty acids, it is convenient to use a secondary standard for routine analyses. The composition of the secondary standard, menhaden oil, is established by analyzing a minimum of 10 replicates at the same time as the primary stan-

dards and using the correction factors calculated from the primary standards to determine composition. FAME are prepared from this secondary standard, in duplicate, with each group of samples analyzed. One replicate is chromatographed prior to the first sample. If the results of the two replicates differ by more than 2% for EPA and DHA, the results of the entire set of samples should be rejected. If the average of the two replicates differs by more than 3% for EPA and DHA from the established composition of the oil, primary standards should be reanalyzed.

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4.4 CONJUGATED OXIDATION PRODUCTS (COP)

INTRODUCTION

The assay for conjugable oxidation products (COP) provides a measure of the extent of oxidative deterioration of polyunsaturated fatty acids (1,2). This assay is utilized in addition to the peroxide and anisidine values to provide a measure of the secondary oxidation products resulting from the further oxidation of hydroperoxides; it also provides insight into which polyunsaturated fatty acids are undergoing oxidative deterioration.

PRINCIPLE

Hydroperoxides are the initial products of autoxidation of unsaturated fatty acids and these highly reactive products may, in turn, give rise to numerous secondary products. In the COP assay, hydroperoxides of polyenoic fatty acids and hydroxy and carbonyl compounds derived from the peroxides, are converted by two sequential chemical reactions into more conjugated chromophores. The reactions used are a reduction followed by a dehydration step. The reaction steps are described in Figure 4.4-1. These products are then characterized by their UV spectra. The spectra distinguish between the oxidation of dienoic fatty acids and that of the more highly polyunsaturated acids because the former yield a conjugated triene chromophore (268 nm) whereas the latter form a conjugated tetraene chromaphore (301 nm).

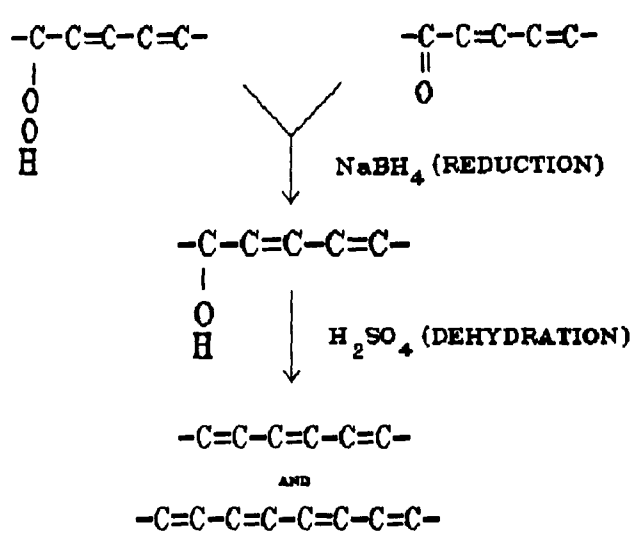

FIGURE 4.4-1. CONJUGATED CHROMOPHORES OF FISH OIL POLYENOIC FATTY ACIDS.

To perform the COP assay, a known weight of lipid is dissolved in isooctane:ethanol and 1 ml aliquots are transferred to each of three flasks. One flask contains the original material taken up to a known volume with ethanol. The second flask contains the same amount of the original material which has been reduced using sodium borohydride and taken to the same volume. The third flask contains the same weight of material, reduced, and dehydrated using a 20% solution of sulphuric acid in ethanol. A UV spectrum is run on the contents of each of the three flasks (Figure 4.4-2). The reduction by sodium borohydride results in the disappearance of characteristic ultraviolet absorbance by carbonyl compounds resulting from the oxidation of polyenoic acids. This decrease in absorbance at 275 nm is defined as the 'oxodiene value'.

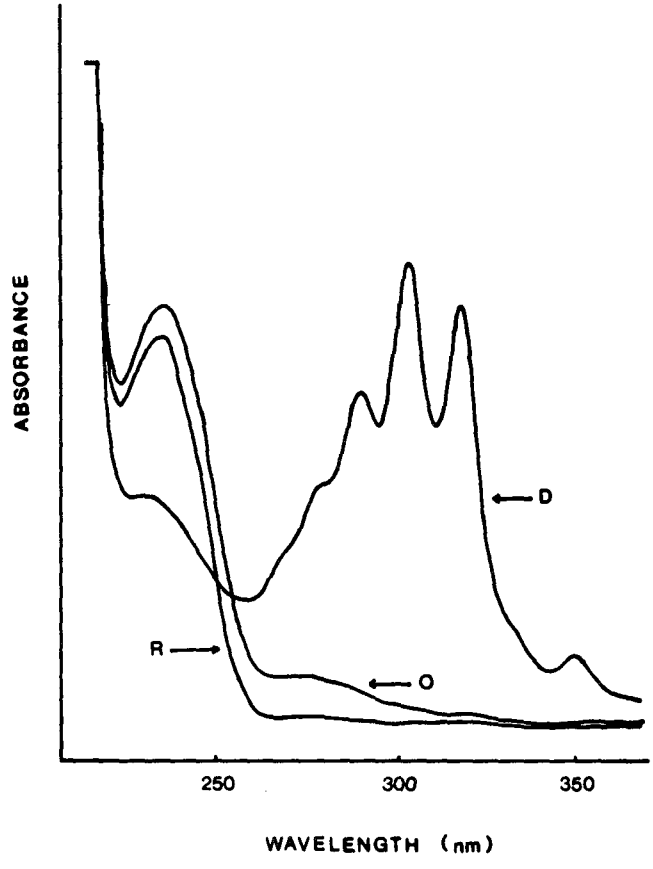

FIGURE 4.4-2. UV SPECTRUM FOR THE DETERMINATION OF COP ASSAY.

Increases in absorbance at 268 and 301 nm during the dehydration step measure the formation of conjugated triene and tetraene chromophores, respectively. The sum of the absorbance increases at these two wavelengths is defined as the conjugable oxidation product value (COP value). The relative proportion of tetraene versus triene products is measured by the ratio of the absorbance change at the two wave lengths (termed the 'COP ratio'). Whereas the ratio is dimensionless, all other numeric values resulting from the COP assay are expressed in units of absorbance for a 1% w/v lipid solution measured in a 1-cm cell.

APPARATUS

* UV spectrophotometer capable of wavelength scanning and recording the scan on a chart (Cary 219 or equivalent)
* Quartz cells, 1 cm matched
* Volumetric flasks, 10 ml and 25 ml
* Water bath, 60°C
* Positive displacement pipette, Mohr type, 1 ml volume
* Filter paper (Whatman No. 1)

REAGENTS

* Isooctane (ACS reagent grade)
* Ethanol, absolute (ACS reagent grade)
* Isopropanol (ACS reagent grade)
* $NaBH_4$ (reagent grade), 1 g in 100 ml isopropanol, filtered through Whatman No. 1 paper to yield a saturated solution (note 1)
* Isooctane:ethanol, 1:1

PREPARATION OF STANDARDS & SAMPLES

1. Weigh approximately 0.5 g oil to the nearest 0.0001 g of oil sample into a 10 ml volumetric flask.
2. Take to volume with 1:1 isooctane:ethanol.
3. Transfer 1 ml aliquots of the diluted sample to each of three 25 ml volumetric flasks, labeled "0" (original), "R" (reduced), and "D" (dehydrated).
4. To "0": Add 1 ml isopropanol and take to volume with ethanol.

   To "R": Add 1 ml $NaBH_4$ and incubate 30 min in a 60°C water bath. Then take to 25 ml with absolute ethanol.

   To "D": Add 1 ml $NaBH_4$ and incubate 30 min in 60°C water bath. Add 5 ml 20% H_2SO_4 in ethanol and incubate 30 min more in 60°C water bath. Take to 25 ml with absolute ethanol.
5. Zero the spectrophotometer with absolute ethanol in each of two matching 1 cm quartz cells, one in the reference beam and one in the sample beam.
6. Scan a 1 ml sample from each flask, over the range of 350 nm to 220 nm, on chart paper calibrated in Absorbance units. Begin with the "0" sample, followed by the "R" sample, followed by the "D" sample. The scans from a single oil sample are performed such that they overlie each other (Figure 4.4-2).

CALCULATION

1. The absorbance at A_{268}, A_{275}, and A_{301} are recorded from the chart for each of the original, reduced and dehydrated samples as A_{268} O, A_{268} R, and A_{268} D.
2. The following calculations are then made:

$$\text{COP value} = (A268D - A268R) + (A301D - A301R)$$

$$\text{COP ratio} = \frac{(A301D - A301R)}{(A268D - A268R)}$$

$$\text{Oxodiene value} = A275O - A275R$$

NOTES

1. $NaBH_4$ may be stored at refrigerator temperature for up to one week. It must be filtered the day of use. Filtration yields a saturated solution of approximately 0.4% w/v.

REFERENCES

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5 ORGANICS

5.1 PCBs AND PESTICIDES

INTRODUCTION

Neither AOCS nor AOAC has an official method for the determination of PCBs or pesticides in marine oils. There are first and final action packed column gas chromatographic methods (1,2) for the determination of organochlorine pesticides and polychlorinated biphenyl residues in fish and non-fatty foods as well as a gel permeation first action method (3) for organochlorine residues in poultry, beef, and swine fats. The method used by the BTM Program for the determination of PCBs and pesticides in oils (4) is an adaptation of methods used by the EPA (5) using capillary gas chromatography and electron capture detection.

PRINCIPLE

The determination of PCBs and pesticides is preceded by the chemical separation of these classes of compounds from other chemical components in the marine oil being analyzed. Gel permeation chromatography is first utilized to separate the PCB/pesticide fractions from the oil on the basis of molecular weight. Florisil adsorption chromatography is then used to separate the PCB/pesticide fractions from other minor components in the oil of similar molecular weight. The sample is then chromatographed simultaneously on two capillary GC columns of different polarity, which serves to confirm identification and quantitation of pesticide and PCB components. The columns are plumbed in the same injection port of the gas chromatograph and each column is plumbed to a different electron capture (EC) detector and integrator. PCBs and pesticides are identified by the retention times of external standards containing a mixture of pesticides or Arochlor 1254. Pesticides are individually quantitated by a microprocessor on the gas chromatograph. For quantitation of PCBs, five representative peaks are identified by the analyst and the integrated peak areas are manually summed as total PCBs in the 1254 suite (7).

APPARATUS

* Column for gel permeation chromatography, 25 cm x 2.5 (Kontes)
* HPLC pump (Laboratory Data Control)
* Rotary evaporator (Brinkmann Buchi RE120 or equivalent)
* Aspirator pump (Cole-Parmer)
* Column for florisil chromatography; 10 cm x 1.0 cm (Tudor)
* Gas chromatograph (Hewlett Packard 5880A or equivalent), equipped with two capillary columns installed in one injection port and plumbed to individual electron capture detectors (EC), and two electronic integrators

* Columns: DB5, 0.25 *um* film thickness, 30m length (J&W Scientific) and
  SPB608, 0.25 *um* film thickness, 30m length (Supelco)
* Rotating sample mixer (Fisher-Kendall or equivalent)
* Analytical balance capable of weighing to 0.0001 g (Mettler AE200 or equivalent)
* Modified 100 ml pear-shaped flask, with 1 ml volumetric tube (Figure 5.1-1) (custom-ordered, Southeastern Laboratory Apparatus.
* Hydrocarbon trap (Chrompak)
* Oxygen trap (Chrompak)
* Moisture trap (Chrompak)

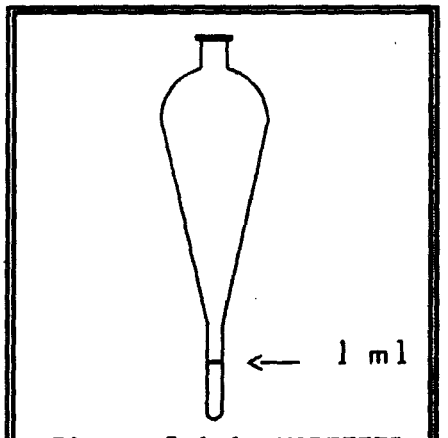

Figure 5.1-1. MODIFIED PEAR FLASK.

REAGENTS

* Biobeads SX-3 (Biorad)
* Methylene chloride:cyclohexane (50:50) (ACS reagent grade)
* Isooctane (ACS reagent grade)
* Florisil, 60/100 mesh (Supelco)
* Petroleum ether:ethyl ether (85:15) (ACS reagent grade)
* Na_2SO_4, anhydrous (Baker)
* Hexane, pesticide grade (ACS reagent grade)
* Arochlor 1254 PCB standard, neat (Environmental Protection Agency)
* MX-7 mixed pesticide standard, in isooctane (Supelco)
* Chlordane standards, neat: *a*-chlordene, *a*-chlordane, *g*-chlordane, transnanochlor, heptachlor (Environmental Protection Agency)
* Hydrogen, ultra-high purity

PREPARATION OF STANDARDS & SAMPLES

I. Standard Preparation

1. Mixed Pesticide Standard:

TABLE 5.1-1. COMPOSITION OF MIXED PESTICIDE STANDARD.

Name	ng/*u*l
a-BHC	0.0090
HCB	0.0045
*b*BHC	0.0360
Lindane	0.0090
Heptachlor	0.0090
a-chlordene	0.0270
aldrin	0.0180
hept EPX	0.0270
g-chlordane	0.0180
a-chlordane	0.0200
transnanochlor	0.0200
dieldrin	0.0450
endrin	0.0720
p,p-DDE	0.0360
o,p-DDD	0.0720
p,p-DDD	0.0720
o,p-DDT	0.0810
p,p-DDT	0.0900

Neat standards are dissolved in isooctane to make concentrated stock solutions. These are stored in glass vials with teflon-lined crimp-top septa at 0°C. MX-7 is obtained in isooctane and is stored as received at 0°C until use. The working standard is made up from concentrated stock solutions in isooctane in a 5 ml volumetric flask. It is then aliquoted into 100 *u*l autosampler vials with teflon-lined crimp-top septa for GC analysis.

2. PCB Standard:

Arochlor 1254 is received in neat form and is dissolved in isooctane to obtain a concentrated stock solution. The concentrated stock is diluted quantitatively to give a standard with a final concentration of 0.08 ng/*u*l.

II. Sample Preparation

1. Mix oil thoroughly on sample mixer.

2. Weigh duplicate samples, between 1.5 - 2.5 g to the nearest 0.0001 g, into individual 25 ml volumetric flasks.

3. Bring each sample to volume with 50:50 methylene chloride:cyclohexane and mix well with a glass disposable pipet rinsed in acetone.

III. **Sample Clean-up**

1. Gel permeation: Gel permeation chromatography is performed on a 25 cm x 2.5 cm column of Biobeads SX-3 eluted with 50:50 MeCl:cyclohexane. A closed system is used (Figure 5.1-2) in which the solvent is pumped onto the column with an HPLC pump and sample is introduced via a sample injection valve with a 1 ml sample loop of polypropylene tubing. Eluent from the column is directed either to a waste jar, to a calibrated pre-run catch flask which allows collection of a known volume of eluent before sample collection begins, or to a sample flask.

   i. Fill the reservoir with eluting solvent. Open the bottom valve (v5) and run the pump for 30 min or until the column packing is totally moistened with solvent.

   ii. To apply sample to the column, fill the sample syringe (a) with 5 ml of sample. With the loop valve in "load" position allow 4 ml of sample to flow through the loop and into the waste bottle (d). Switch the loop valve to "column" position, to direct the 1 ml content of the loop onto the column. Simultaneously close the stopcock (f), at the bottom of the pre-run catch flask.

   iii. The pre-run cut point is 79 ml. Once the forerun has eluted, switch v2 located at the column exit, to direct the eluting material to a pear-shaped flask (h). Elute 100 ml of solvent into the pear-shaped flask, as determined from flow rate calculated by timing the collection of 79 ml pre-run. Upon completion of the elution, switch the outlet valve (v2) back to its original position.

   iv. After sample is eluted, back-flush the column to prepare it for the next sample. Turn pump off and wait until pulse damper is empty. Then turn valves 1,3, and 4 to reverse flow of the solvent through the column. Turn pump on and run for 5-10 min. Store column with valve 5 in the closed position to keep eluting solvent on the column after all valves are returned to original position.

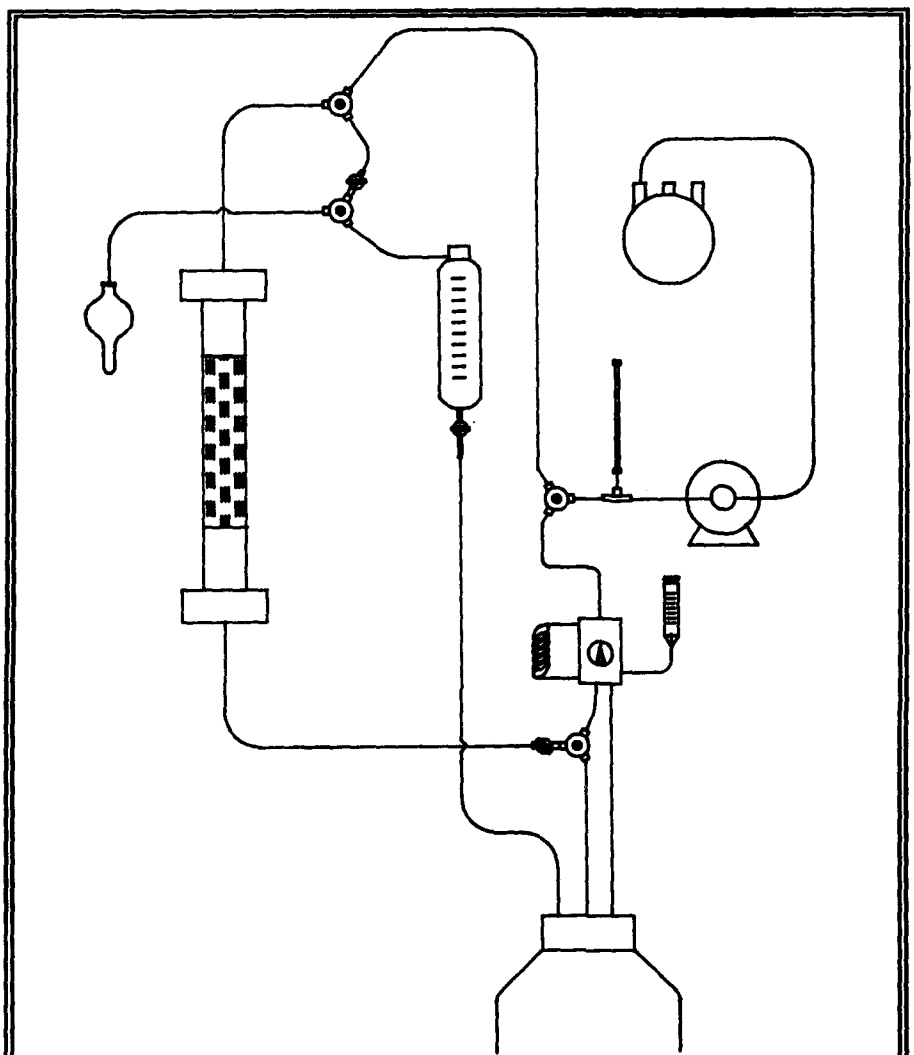

FIGURE 5.1-2. GEL PERMEATION COLUMN FOR PESTICIDE/PCB SAMPLE CLEANUP.

2. Rotary evaporation: Evaporate the eluting solvent (100 ml) down to 0.5 ml in the pear-shaped flask using an aspirator pump as the source of vacuum. Be certain the sample does not go to complete dryness. Water bath temperature should be 40°C. Spin rate should be 5. Add an additional 0.5 ml of isooctane and continue evaporation down to a volume of 0.5 ml. Repeat the last process 3 times. Following the last evaporation, take to 1 ml with isooctane, cap the sample.
3. Florisil chromatography: Fill a column with florisil to 9.4 cm and activate the column overnight in a 130°C oven. After cooling the column in a desiccator, place it in an appropriate holder and add 1.0 cm of hexane-extracted Na_2SO_4 (Note 1). Wet the column packing with 20 ml 85:15 petroleum ether:ethyl ether. Do not allow the column packing to go dry; keep the solvent level at the top of the Na_2SO_4 layer by closing the stopcock when the solvent is about 1 cm above the

Na_2SO_4. Transfer the 1 ml sample from the pear-flask to the column using a disposable glass Pasteur pipet. Open stopcock to begin elution of the sample. Follow the transfer with two 1 ml washes of the sample flask with isooctane. When the added sample on the the column reaches the top of the Na_2SO_4 layer, add an additional 5 ml of isooctane to wash the walls of the column. Following sample application, elute with the remaining solvent (approximately 35 ml). The elution volume varies with each lot of florisil, which must be calibrated using standard methodology (Note 2). The column flow should be 4-5 ml/min; it may be necessary to pressurize with nitrogen to attain this flow rate. The eluent is collected in a modified pear-flask (Figure 5.1-1).

4. Rotary evaporation: Add 1 ml of isooctane to the eluent from the florisil column and process as described in step 2. Evaporate to a volume of 1.0 ml using the 1.0 ml volumetric tube on the pear-shaped flask to measure. Transfer approximately 0.1 ml to a 100 *u*l crimp-top glass autosampler vial with a teflon-lined septum. The sample is ready for GC analysis at this stage. The remainder of the sample is stored in a 2 ml glass crimp-top vial at -20°C.

DETERMINATION

Samples are chromatographed simultaneously on two capillary GC columns of different polarity plumbed into the same injection port, using a two-hole ferrule. The DB-5 column is used for quantitation. The SPB608 column is used for confirmation of peak identifications and quantitations. Since quantitation is based on external standards, the use of an autosampler is recommended to obtain the necessary precision in sample volume. Temperature programming parameters used are listed in Table 5.1-2. Flow rate is set manually at about 1 ml/min. The carrier gas is ultra high purity hydrogen, which is further cleaned by in-line moisture, oxygen and hydrocarbon traps.

TABLE 5.1-2. GC TEMPERATURE PROGRAM PARAMETERS FOR THE SEPARATION OF PCB/PESTICIDE FRACTIONS.

Detector temp	350°C
Injection temp	250°C
Oven temp profile	
initial value	90°C
initial time	0.50 min
level 1	
prgm rate	5.00°C/min
final value	165°C
final time	0.00 min
level 2	
prgm rate	0.10°C/min
final value	172°C
final time	0.00 min
level 3	
prgm rate	1.00°C/min
final value	220°C
final time	60.00 min
post value	230°C
post time	15.00 min

1. Sample vials are placed in an autosampler in odd-numbered sample slots. The even-numbered sample slots contain wash vials filled with isooctane. A PCB and a pesticide standard are run after every three samples in order to assure correct quantitation. 2 *u*l samples are injected.
2. On the HP5880 gas chromatograph, the user enters the sample "ID"s into a "Sample Table". When a sample slot is identified as a pesticide standard, the instrument invokes an automatic recalibration of its response factor for each pesticide.

CALCULATION

1. **Pesticides:** The identification and quantitation of pesticides is done entirely by the microprocessor on the HP5880 GC by comparison of peak retention times with those in the standard, calculation of peak area, and calculation of ppm of unknowns using the peak area response determined from the standard and a multiplier entered into the sample table for each sample by the user. The multiplier takes into account the original sample weight and the volume chromatographed.

$$\text{ppm unknown} = \frac{\text{peak area (sample)}}{\text{peak area (std)}} * \text{ng std} * \text{multiplier}$$

where,

$$\text{multiplier} = \frac{1}{2\ \text{ul}} \cdot \frac{25\ \text{ml}}{\text{sample wt}} \cdot \frac{1}{1.27}$$

Two *u*l is the sample volume chromatographed, 25 ml is the original sample volume, and 1.27 is a correction factor correcting for the fact that 1.27 ml of the original sample is actually applied to the gel permeation column in the first step of sample clean-up, rather than 1 ml in the sample loop, due to the volume of tubing leading to the column. This correction factor must be determined in each individual laboratory.

The analyst must confirm the identification of peaks by comparing the identifications made by the instrument on the DB-5 chromatogram with those made on the confirmation column. If a component identified as a pesticide in the DB-5 chromatogram is missing in the SPB608 chromatogram, the identification is considered an error.

2. **PCBs:** Identification and quantification of PCB components is made by the analyst. First, area measurements of 5 representative peaks present in the Arochlor 1254 standard (7) are summed. The areas of the 5 corresponding sample peaks identified by the analyst as 1254 components are then summed and total PCBs in the sample are quantitated:

$$\text{ppm total PCBs} = \frac{\text{summed peak area (sample)}}{\text{summed peak area (std)}} \cdot \text{ng std} \cdot \text{multiplier}$$

where,

$$\text{multiplier} = \frac{1}{2\ \text{ul}} \cdot \frac{25\ \text{ml}}{\text{sample wt}} \cdot \frac{1}{1.27}$$

Two *u*l is the sample volume chromatographed, 25 ml is the original sample volume, and 1.27 is a correction factor correcting for the fact that 1.27 ml of the original sample is actually applied to the gel permeation column in the first step of sample clean-up, rather than 1 ml in the sample loop, due to the volume of tubing leading to the column.

The identification of PCB peaks is confirmed by comparison of the DB-5 chromatogram with the SPB608 chromatogram. If a peak identified as a PCB in the DB-5 chromatogram is missing in the SPB608 chromatogram, the identification is considered an error.

PRECISION

Duplicate samples of oil carried independently through the sample preparation procedure and gas chromatography analysis should have a relative standard deviation of less than 15%. The mean recovery of PCBs spiked at 0.05-0.1 ppm in menhaden oil is 101.5%.

NOTES

1. Cleaning of Na_2SO_4 by extraction with hexane in a Soxhlet extraction apparatus: Place approximately 100 g Na_2SO_4 in pyrex soxhlet thimble. Place the thimble in the soxhlet apparatus. The reflux should be cooled by running cold water in the jacket. Place boiling chips and 300 ml hexane in a round botton flask and attach to the bottom of the soxhlet apparatus. Heat the hexane until boiling using a heating mantle plugged into a variac and allow to reflux 8 h. Evaporate hexane in hood 1-2 hr until dry. Place dry Na_2SO_4 in an oven at 130°C. Store in oven until use. Before use in the florisil column, allow the Na_2SO_4 to come to room temperature in a dessicator.
2. Calibration of florisil by lot: Because each lot of florisil varies in its adsorbent activity, each new lot of florisil must be calibrated to determine the volume of eluting solvent required to remove the PCBs and pesticides. Prepare a florisil column as in the sample clean-up procedure above. Elute a 10-fold concentrated PCB and pesticide standard, collecting three 10-ml fractions followed by 20 2-ml fractions. Analyze each fraction for PCBs and pesticides by GC as described above. Determine at what elution volume the standards are no longer present. This determines the elution volume used in the florisil chromatography step above.

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5.2 UREA

INTRODUCTION

The AOAC has several classical tests for urea including the Urease Test for Urea (#44.164)(1) and the Xanthydol Test for Urea (#44.165) (2). These tests usually require an analyst skilled in microcrystal testing. None of the methods are directly applicable to the estimate of urea residues in fatty acid esters. The method utilized by the BTM Program is a modification of a spectrophotometric method developed for the determination of suspected urea in urine stains on foods and containers (3), and was chosen by the project because it is sensitive to the ppm level, does not require an analyst skilled in microcrystal testing, and is based on an initial extraction of the material to be tested.

PRINCIPLE

Urea is extracted from esters of fish oil into distilled water. The urea assay is performed on the water extract. The procedure is based on the formation of ammonia from the reaction of urea with urease followed by the formation of a blue compound, indophenol, by the reaction of ammonia and phenol in the presence of hypochlorite. Indophenol is quantified by spectrophotometry. A calibration curve is constructed using urea standards of several concentrations versus their absorbance at 625 nm. The concentration of urea in samples is determined by linear regression analysis using the calibration curve constructed.

APPARATUS

* Culture tubes, 125 mm x 25 mm
* Automated pipet, positive displacement, 1 ml
* Counter top centrifuge (capable of attaining 1000 x g)
* Spectrophotometer (capable of measuring $\pm$ 0.001 Abs units)
* Glass cuvettes, 1 cm
* Volumetric tubes, 10 ml

REAGENTS

* Chloroform (Reagent grade)
* Urease (Sigma), 10 mg/ml in distilled water
* Manganous sulfate, reagent grade, 0.05%
* Urea, reagent grade, 1.0 g/L acetone stock solution

* Hypochlorous acid: add 5 ml 5.25% commercial bleach to 20 ml distilled H_2O. Adjust to pH 6.5-7.0 with HCl.
* Phenol solution: 0.5 g NaOH and 2 g phenol in 20 ml distilled H_2O

PREPARATION OF STANDARDS & SAMPLES

All samples, procedural blanks and spiked samples are prepared and analyzed in duplicate. The procedural blank (Note 1) is an empty tube that is carried through the entire procedure. The spiked sample (Note 2) is included to determine the % recovery from the extraction procedure.

I. **Extraction of urea from ethyl esters of fish oil:**

1. Weigh 0.5 g esters into a culture tube.
2. Add 3 ml $CHCl_3$.
3. Add 1 ml distilled H_2O.
4. Extract by mixing on Vortex mixer 15 sec.
5. Centrifuge tube 15 min at 1000 x g.
6. Remove water layer and transfer to 10 ml volumetric centrifuge tube.
7. Repeat extraction procedure (3-6) two more times.
8. Back-wash the combined water extracts with 1 ml $CHCl_3$ by mixing on Vortex 15 sec, centrifuging 15 min at 1000 x g and removing the $CHCl_3$ layer.
9. Take the combined water layers to 10 ml in a volumetric tube.

II. **Preparation of Standard Curve**

1. The assay is linear from 3-15 *ug* urea/ml. A standard curve of 0,3,6,9,12, and 15 ug/ml urea is prepared from the urea stock solution above by pipetting 0, 30, 60, 90 120 and 150 *u*l stock solution into 10 ml volumetric tubes, evaporating the solution to dryness under nitrogen, and bringing to 10 ml with H_2O.

DETERMINATION

1. To each standard, blank and sample tube, add 3 drops urease solution.
2. Heat in hot water bath at 55°C for 30 min.
3. Cool to room temperature.
4. Add 1 drop $MnSO_4$.
5. Add 10 drops hypochlorous acid solution.

6. Immediately add 12 drops phenol solution. Incubate at room temperature for 10 minutes.
7. Transfer 1 ml of each solution to 1 cm cuvette and read absorbance at 625 nm.

CALCULATION

Create a standard curve of urea concentration versus absorbance. Determine the concentration of urea in the unknown samples by linear regression using the standard curve constructed. Samples must be diluted to a concentration that falls within the linear range of the standard curve.

PRECISION

Samples analyzed in duplicate should have less than a 10% relative standard deviation.

NOTES

1. Color intensity is affected by age of the reagents; therefore, a procedural blank and/or control should be carried through the entire procedure. The procedural blank consists of an empty tube which is carried through all extraction and determination steps as a sample.

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5.3 ANTIOXIDANTS

5.3.1 TERT-BUTYL HYDROQUINONE (TBHQ)

INTRODUCTION

The BTM Program utilizes the antioxidant tertiary butyl hydroquinone (TBHQ) in many of the test materials produced at the Charleston Laboratory. The FDA set a legal limit for the concentration of TBHQ in food products at 0.02%. To assure the concentration of antioxidant added, the QA/QC Project utilizes a gas chromatography method for the analysis of phenolic antioxidants in vegetable oils (1). The method allows simultaneous analysis of (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) and can successfully be applied to fish oils. The BTM Program utilizes a slight modification of the published method, which simplifies analysis of TBHQ.

PRINCIPLE

For analysis of fish oil, the antioxidants are extracted into acetonitrile containing an internal standard, propyl paraben (propyl parahydroxybenzoate). A silyl derivative is then made of the extracted sample. Derivatization is necessary for the analysis of TBHQ in order to stabilize it in the reduced hydroquinone form. PG will not elute in the underivatized form under the conditions employed. BHA and BHT may be analyzed in their underivatized forms using GLC equipped with flame ionization detection. The derivatized extracts are separated on a megabore wall coated open-tubular (WCOT) column and quantified using an internal standard.

APPARATUS

* Volumetric flasks (2), 50 ml.
* Automated positive displacement pipet, 0.2-1.0 ml (SMI or equivalent).
* 16 x 125 mm test tubes with teflon-lined screw caps.
* Gas chromatograph equipped with flame ionization detector and electronic integrator (HP5840 or equivalent).
* Analytical balance capable of weighing $\pm$0.001 g (Mettler AE200 or equivalent).
* Megabore WCOT column, 0.53 mm (J & W Scientific, DB-5 or equivalent).
* Hamilton syringe, 10 *u*l (or equivalent)

REAGENTS

* Acetonitrile (ACS reagent grade).
* Propyl paraben (Sigma), 200 *ug*/ml in acetonitrile.
* Tertiary butyl hydroquinone (Kodak), 250 *ug*/ml in acetonitrile.
* N,O-bis-(trimethylsilyl) trifluoroacetamide, BSTFA (Pierce).

PREPARATION OF STANDARDS & SAMPLES

1. **Calibration Standard:**

Add 1 ml propyl paraben stock solution and 1 ml acetonitrile to 1 ml TBHQ stock solution. This standard is used to calculate a GC response factor for TBHQ.

2. **Extraction of TBHQ from oil:**

   a. Weigh 1 g oil (to the nearest 0.001 g) into a 16 x 125 mm tube.
   b. Add 1 ml of 200 mg/ml propyl paraben in acetonitrile.
   c. Shake by hand 15 sec.
   d. Centrifuge in counter top centrifuge 3 minutes to separate phases.
   e. Remove acetonitrile layer to a second tube.
   f. Add 1 ml acetonitrile (not containing propyl paraben).
   g. Shake 15 seconds.
   h. Centrifuge 3 minutes.
   i. Remove acetonitrile layer to another tube.
   j. Repeat extraction procedure once (f-i).

3. **Silyl derivatization:**

   a. To each extracted sample and the calibration standard add 0.25 ml BSTFA.
   b. Mix thoroughly and place in a 80-90°C water bath for 20 min.
   c. Remove and allow to come to room temperature.

DETERMINATION

The following parameters are used for analysis of the derivatized samples by gas chromatography (Table 5.3.1-1).

TABLE 5.3.1-1. GC PARAMETERS FOR THE DETERMINATION OF TBHQ.

Injection temperature	250°C
FID temperature	250°C
Oven temperature	150°C
Stop	15 min
Flow Rate	30
Attenuation	2^3
Chart Speed	0.25

1. Make injections of 1 *u*l derivatized calibration standard or sample using a 10 *u*l syringe. Analyses are performed in duplicate.

CALCULATION

1. **GC Response Factor.** The GC calculates a relative response factor for TBHQ using the following equation:

$$\text{Response factor (TBHQ)} = \frac{\text{amount sample}}{\text{area sample}} \cdot \frac{\text{area internal standard}}{\text{amount internal standard}}$$

By entering the known weights of TBHQ and the internal standard, the GC determines the response factor which will be used later for the calculation of TBHQ in the samples. The response factor for the internal standard is defined as 1. This calibration is run in duplicate.

2. **TBHQ in Oil.** The HP5840 GC uses the following equation to determine the amount of TBHQ in a sample:

$$\%\ \text{TBHQ} = \frac{\text{area (sample)} \cdot \text{response factor (sample)}}{\text{area (IS)} \cdot \text{response factor (IS)}} \cdot \frac{\text{weight (IS)}}{\text{weight (sample)}} \cdot 100$$

PRECISION

Duplicate analyses on a sample should have a relative standard deviation of less than 3%.

REFERENCES

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5.3.2 TOCOPHEROLS

INTRODUCTION

Alpha- and gamma-tocopherols are routinely added to many of the BTM Program test materials for their antioxidant capacity. The test materials containing tocopherols are analyzed to assure the levels added. Alpha- and gamma-tocopherols are also analyzed in placebo vegetable oils to provide researchers with information on naturally occurring quantities present or quantities added when test materials and placebos are matched for antioxidant content.

Official methods for gas chromatographic (1) and colorimetric (2) determination of alpha-tocopherol have received final action by the AOAC. Neither method provides for the determination of gamma-tocopherol . The method used by the BTM Program for fish oils and esters of fish oils is a modification of methodology developed at the USDA Human Nutrition Research Center in Beltsville, MD (3) that permits determination of cholesterol, major plant sterols, and alpha- and gamma-tocopherols from a single analysis (also see section 3).

PRINCIPLE

A sample containing approximately 0.1 g of lipid (fat, oil or lipid extract) is mixed with a known weight of the internal standard (5-*a*-cholestane) and saponified at 80°C with aqueous KOH in the presence of pyrogallol. The unsaponifiables are extracted with cyclohexane, the solvent is removed under nitrogen, and trimethylsilyl ethers are prepared using N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane in pyridine. This derivatized, total unsaponifiable fraction is chromatographed on a non-polar fused silica wall-coated open-tubular (WCOT) column. Cholesterol, alpha- and gamma-tocopherol are identified by comparison of peak retention times with that of an internal standard and with external tocopherol standards prepared and chromatographed in an manner identical to the samples. Linear regression equations derived from analysis of the external standards are used to calculate the amount of each component in the samples.

APPARATUS

* Gas chromatograph (HP5840 or equivalent), equipped for use with capillary columns (0.22-0.32 mm id), with an FID detector and optional autosampler
* Non-polar flexible fused-silica capillary column, 25m x 0.2mm x 0.3*u* film thickness (Hewlett-Packard Ultra 2) or 50m x 0.22mm x 0.25*u* film thickness (J&W Scientific DB-1)

* Centrifuge (Centra 4 International Centrifuge or equivalent) with rotor to accomodate 25 x 150 mm screw cap tubes and operate at 1300 rpm
* Nitrogen evaporator with water bath capable of 37°C (Meyers N-Evap or equivalent)
* Water bath, 80°C (Precision Scientific or equivalent)
* Vortex mixer
* Pasteur pipettes, disposable
* Automatic positive displacement pipettor capable of delivering 1 ml ±0.01 ml (Eppendorf Repeater Pipet Model No. 2226 000-6 or equivalent)
* Centrifuge tubes, 25 x 125 mm, with teflon lined screw caps
* Disposable culture tubes, 16 x 125 mm, with teflon lined screw caps, silanized
* Analytical balance (Mettler AE163 or equivalent)
* Mechanical sample mixer (Fisher-Kendall or equivalent)
* Moisture trap (Chrompak)
* Oxygen trap (Chrompak)
* Hamilton syringe, 10 ul (or equivalent)

REAGENTS

* Hexane (ACS grade)
* Isooctane (ACS grade)
* Butylated hydroxytoluene (BHT) (Sigma)
* Nitrogen, compressed
* KOH (reagent grade), aqueous saturated solution (prepare fresh daily)
* Pyridine, silation grade (Pierce Chemical Co.)
* N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (Supelco Sylon BFT or equivalent)
* Sylon CT (Supelco) (Note 1)
* Alpha-tocopherol, gamma-tocopherol, in hexane in sealed ampules (Supelco)
* 5-*a*-Cholestane (Supelco)
* Absolute ethanol, reagent grade, degassed (Note 2)
* Pyrogallol, reagent grade (Malinckrodt), 3% w/v in degassed absolute ethanol (note 2)
* Helium, 99.999% pure
* Cyclohexane, degassed (Note 2)

PREPARATION OF STANDARDS AND SAMPLES

Tocopherols can be destroyed by sunlight and white fluorescent light, especially under alkaline conditions. This method is carried out under yellow fluorescent lighting. Subdued incandescent lighting may be used if all windows are covered to exclude sunlight.

I. Standards

1. **Internal standard:** 40-50 *ug*/ml 5-*a* cholestane + 20 *ug*/ml BHT in isooctane.
2. **Mixed tocopherol stock standard:** Prepare appropriate dilutions of alpha- and gamma-tocopherols in isooctane. Store in amber glass at -10°C. From the stock standard, make three different dilutions in isooctane which span the expected range of concentrations to be found in samples being analyzed.

II. Samples

1. Weigh approximately 100 mg of oil or ester concentrate (to the nearest 0.0001 g) into a 50 ml screw capped centrifuge tube.
2. Add 1.0 ml internal standard to each sample and tocopherol standard.
3. Evaporate the solvent using the 37°C water bath.
4. Add 8 ml pyrogallol-absolute ethanol solution and 0.5 ml saturated KOH.
5. Cap tubes tightly and vortex 5 sec.
6. Heat tubes 8 min in 80°C waterbath; shake vigorously after 1,2 and 4 min so that the solution washes tube sides and caps.
7. Remove tubes and cool in cold water for 15 sec.
8. Add 10 ml cyclohexane and vortex for 5 sec.
9. Add 6 ml distilled water and shake for 15 min (mechanical mixer).
10. Centrifuge for 15 min (1300 x g).
11. Transfer cyclohexane layer to a 16 x 125 mm silanized culture tube.
12. Place tubes in N-Evap water bath at 37°C and evaporate the cyclohexane under a gentle stream of nitrogen.
13. Add 100 *u*l pyridine and 100 *u*l Sylon BFT; vortex 5 sec and let stand 15 min at room temperature. The sample is now ready for analysis by GC.

DETERMINATION

The extracted samples are chromatographed isothermally on a fused silica capillary column (DB-1 or HP Ultra 2) using an all-glass split injection port with a split ratio of approximately 1:40. Carrier gas is helium (99.99% pure) with in-line moisture and oxygen traps. The average linear velocity is approximately 30 cm/sec. Nitrogen is used as the make-up gas to the flame ionization detector (FID). The combined flow rate at the detector (carrier + makeup) is 30 ml/min. The following temperature parameters are used on the HP5840 GC: injection port - 300°C; detector - 300°C; column oven - 280°C.

A 1 *ul* sample is injected, either manually using a 10 *ul* syringe, or by autosampler (Note 3).

CALCULATION

Retention times are calculated for the tocopherol standards relative to that of the internal standard. The tocopherol peaks are then identified in the sample by comparison of the relative retention time with those of the tocopherol standards.

A calibration curve is created for each tocopherol by analyzing at least three concentrations of tocopherol standards, where x = the weight ratio and y = peak area ratio of the tocopherol standard to the internal standard. Determine the slope (m) and the y-intercept (a) for the linear regression.

The tocopherol peaks in the sample are quantitated by the formula:

$$mg/g = \left(\frac{A(t)}{A(i)} - a\right) \cdot \frac{W(i)}{m \cdot W(s)}$$

W(i) = weight of internal standard, mg
A(i) = area of internal standard
A(t) = area of component T
W(s) = sample weight, g
m = slope
a = y-intercept

PRECISION

The analysis is precise at ± 2% for duplicates.

NOTES

1. The purity of standards should be evaluated by TLC and GC (Sections 2.1 and 2.2 of this manual).

2. Degassing solvents:

   a. Water - boil 15 min. Cool. Stopper tightly and store at room temperature.

   b. Cyclohexane and absolute ethanol - degas under vacuum using a rotary evaporator.

3. Sylon BFT is corrosive and will attack syringe needles and plungers; thus they should be rinsed promptly after exposure. Pryidine will cause some elastomers to swell (e.g., vitons). Glass and teflon are resistant to pyridine. TMS ethers are unstable if stored in BFT. To preserve the derivative, add 1 ml of petroleum ether and remove solvent and reagent with a gentle stream of nitrogen. Dissolve residue in isooctane.

REFERENCES

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6 METALS

INTRODUCTION

The BTM Program performs analyses for a wide array of metals in the fish oil test materials produced by the BTM Program. The metals selected for analysis are those that may either be native to the oil or introduced during processing. These include the potentially toxic metals (Hg, Cd, As, Se, Pb, Ni, and Cr) and elements of nutritional importance in a biological test material (including Ca, Cu, Fe, K, Na, and Zn). The methods for conducting the analyses were selected based on the need for time efficiency, both in terms of the time required for sample preparation and for analysis. The methods employed do not necessarily provide the lowest possible detection limit, but provide detection limits well below FDA or international regulatory limits imposed on fish and fishery products. Hg is the only metal regulated in fishery products by the FDA, with a limit of 1.0 ppm as CH_3Hg. Other countries impose various limits on As, Cd, Cr, Cu, Hg, Pb, Sn, Se, and Zn contents of fishery products, generally above 1 ppm, but occasionally as low as 0.1 ppm for As, Cd, and Hg.

Standard methods are not currently available for the determination of metals in fish oils. The AOCS published a tentative method for the analysis of Cr, Cu, Fe, Ni, and Mn in vegetable oils using methyl isobutyl ketone (MIBK) extraction and analysis by flame atomic absorption spectrophotometry (1, Section 6.1) and graphite furnace (2, Section 6.1). The MIBK extraction coupled with flame AA is sensitive only to 2.0 ppm of each metal and therefore not suitable for our purposes. The BTM Program utilizes microwave digestion of oil samples followed by analysis using either flame AA, hydride generation, or polarography.

6.1 DIGESTION PROCEDURES

INTRODUCTION

A number of wet digestion methods are available for removing organic components from samples of biological origin (3,4). Oil samples present a particularly reactive matrix that make many of the conventional methods time consuming and/or dangerous. The BTM Program employs a microwave digestion technique using closed teflon digestion vessels. This method is comparatively rapid, requiring several hours digestion time, as opposed to up to two weeks for digestion using conventional wet digestion techniques.

PRINCIPLE

The most commonly used methods for preparing biological matrices for metals analysis are a variety of wet acid digestion techniques (3,4). Most methods use a combination of nitric acid and another acid with stronger oxidizing power. Nitric acid readily reacts with both aromatic and aliphatic compounds. It rapidly oxidizes aliphatic polyhydroxyl compounds, degrading them to simple carboxylic acids (3). Nitric acid digestion of fish oils in open vessels yields an incomplete digestion, leaving a solid waxy residue even after an extensive digestion of 96 hours. This residue is believed to consist of paraffins which are the saturated products of the incomplete digestion. Complete digestion of fish oils using nitric/perchloric/sulfuric acids in micro Kjeldahl flasks can take up to two weeks. The digestion of samples in closed vessels improves the performance of the acids by increasing the temperatures achieved (5,6). Heating closed vessels by microwaves further enhances the performance of digestion by decreasing the time required to reach the desired temperatures. Microwave digestion techniques are relatively new; thus, no standard methods exist.

In the method developed at the Charleston Laboratory, fish oils or esters of fish oils are first allowes to pre-digest in nitric acid at room temperature in open teflon microwave digestion vessels. The samples are then microwave digested with nitric acid in closed vessels for two hours. An incomplete digestion results, which is similar to that obtained after 96 hr of digestion in micro Kjeldahl flasks described above. After digestion with nitric acid, a small amount of sulfuric acid is added to the sample and digestion is continued for one hour. The vessels are then opened, the contents evaporated to approximately 1 ml, and 30% hydrogen peroxide is added, 1 ml at a time, until a clear to very pale yellow digestate remains, with a volume of approximately 1 ml. The samples are then taken volumetrically to 10 ml with distilled H_2O.

The microwave digestion system used consists of 120 ml teflon vessels with screw-on lids that possess a pressure release valve which opens at 120 psi. The vessels are capped using an automated "capping station" which achieves the same torque on all caps, ensuring that the release valve will open at the specified pressure. The vessels fit into a turntable and each vessel is vented into an overflow container containing solid sodium bicarbonate to help neutralize the acid fumes released from the vessels. The microwave oven is equipped with an exhaust tube which is vented into a laboratory hood.

All sample digestions are accompanied by a blank, a spiked fish oil or ester sample and NBS standard reference materials (oyster or bovine liver) in order to determine recovery and to ensure accuracy of the analyses.

APPARATUS

* Analytical balance (Mettler AE200 or equivalent)
* Automatic pipet (Rainin Pipetman 1000 or equivalent)
* Microwave digestion oven (CEM Corporation), equipped with vessel capping station, turntable and twelve closed teflon digestion vessels with pressure relief valves (Note 1)
* Volumetric flasks, 10- and 25 ml
* Nalgene bottles, 30 ml acid washed

REAGENTS

* Nitric acid (Ultrex grade, J.T. Baker)
* Sulfuric acid (Ultrex grade, J.T. Baker)
* Hydrogen peroxide (Ultrex grade, J.T. Baker)
* Sodium bicarbonate
* NBS standard reference material (oyster or bovine liver)
* Reference metal standards, 1000 ug/ml, for each metal of interest (Fisher or equivalent)

PREPARATION OF STANDARDS AND SAMPLES

1. Weigh 0.5 g NBS standard or 1 g (to the nearest 0.0001 g) fish oil or ester into a teflon digestion vessel. For spiked oil/ester samples, add 0.1 ml of a 100 ug/ml mixed metal stock solution. For blanks, use an empty vessel.
2. Add 15 ml of nitric acid. Swirl.
3. Place lid on the teflon vessel and tighten the lid using the capping station to achieve proper torque.

4. Place all vessels (up to 12) in the turntable of the microwave oven, being certain to connect vent tubes to the venting trap. The trap should contain solid Na_2HCO_3 approximately one-half inch in depth to neutralize fumes vented from the vessels.

5. Begin heating the oven, 50% power, manually venting the vessels every two minutes for the first ten minutes. Continue heating, venting the vessels every five minutes for the next 20 minutes. Continue heating for 1 1/2 hours more, venting manually every 15 minutes.

6. Remove vessels from the oven, allow to cool to room temperature. A cool water bath can be used to speed the cooling process.

7. Remove the caps using the capping station. Add 1 ml sulfuric acid. Replace caps and tighten to the proper torque with the capping station.

8. Replace the vessels in the turntable and place the turntable in the oven. Heat on 50% power for 5 min. Manually vent the vessels. Continue heating for one hour, manually venting every 20 min.

9. Remove vessels from oven. Remove caps. Rinse insides of caps with a small volume of nitric acid into the vessels.

10. Replace the vessels, without lids, to the turntable. Place turntable in oven. Heat on 30% power to evaporate. This may take 1-2 hr. As the nitric acid evaporates, the digestate will become darker in color. However, avoid allowing the sample to char. (This will result in lower recoveries of some metals.)

11. Add hydrogen peroxide to the hot samples, 1 ml at a time, until nearly all color is removed from a sample which is no more than 1 ml in volume.

12. To test if a digestion is completed, add 1 ml distilled H_2O to the cooled digest. If the sample becomes cloudy, add nitric acid and continue heating followed by addition of hydrogen peroxide until a complete digestion is obtained.

12. Transfer cooled digests to 10 ml volumetric flasks and take to volume with 2% nitric acid. Transfer diluted samples to 30 ml Nalgene bottles for storage until analyzed.

NOTES

1. Acid washing of glass, teflon and plastic can be accomplished by soaking clean glassware in a nitric acid bath containing 1:3 H_2O:concentrated HNO_3 (reagent grade). After acid-soaking, acid washed items should be rinsed in distilled water, dried, and stored in clean, covered cabinets.

REFERENCES

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6.2 FLAME ATOMIC ABSORPTION (Ca, Cr, Cd, Cu, Fe, K, Na, Ni, Zn)

INTRODUCTION

Flame atomic absorption (AA) spectroscopy is used to analyze Cd, Cr, Ca, Cu, Fe, K, Na, Ni, and Zn in acid digested fish oil and ester samples. For many of these elements, flame AA is not the most sensitive method available, but it is the least time consuming and is of adequate sensitivity for the purposes of quality assurance of the biological test materials from the standpoint of determining the safety and nutritional composition of the oil.

PRINCIPLE

Metals in the original oil or ester samples are present in the digested sample in their elemental forms. Nitric acid serves to maintain the elements in solution, minimizing binding of the elements with glass in which the samples are diluted or plastic in which the samples are stored. The basis of flame AA is that each element absorbs light of specific wavelength(s). A hollow cathode lamp is used as a source of light of a specific wavelength. The light is detected by a wavelength specific detector. A sample containing the element of interest is aspirated into a flame located in the path of the light beam. The detector determines what portion of the incident light is absorbed by the sample. A linear relationship exists (Beer's Law) between the concentration of the absorbing element and the attenuation of the light beam. A calibration curve of concentration versus absorbance is created using standards containing each element of interest. The samples, standards and blanks are then analyzed and concentrations of the element are calculated by linear regression analysis using the calibration curve.

APPARATUS

* Flame atomic absorption spectrophotometer, dual beam, with background correction capability, equipped with an air/acetylene burner head (IL751 or equivalent)
* Acetylene, atomic absorption grade
* Compressed breathing air
* Single element hollow cathode lamps Ca, Cr, Cd, Cu, Fe, K, Na, Ni, Zn (Fisher or Instrumentation Laboratories)
* 50 ml volumetric flasks, glass or polypropylene
* 25 ml volumetric flasks, glass or polypropylene
* 5 ml, 1 ml, and 0.2 ml automated pipets and tips (Rainin Pipetman or equivalent)
* Automatic repeating pipet (Eppendorf Repeater Pipet or equivalent)
* 5 ml polypropylene test tubes with caps (Sarstedt), acid washed

* Nalgene bottles, 30 ml acid washed

REAGENTS

* 1000 *ug*/ml metal standard solutions: Ca, Cd, Cr, Cu, Fe, K, Na, Ni and Zn (Fisher or equivalent)
* Ultrex HNO_3, 2% v/v in distilled H_2O (J.T. Baker)
* La_2O_3, reagent grade (Fisher), 1% w/v in 2% Ultrex HNO_3

PREPARATION OF STANDARDS & SAMPLES

I. **Standards:** For each element a 5-point standard curve is prepared by quantitatively diluting commercially prepared 1000 *ug*/ml standard solutions in 2% Ultrex HNO_3. Standards are made up in 25 ml volumetric flasks and transferred to 30 ml acid washed Nalgene bottles for storage. The standard concentrations used depend upon the detection limits and linear working range for each element and the concentration of each element anticipated in the oil or ester samples (Table 6.2-1).

TABLE 6.2-1. METAL STANDARDS FOR FLAME ATOMIC ABSORPTION.

	Concentration (*ug*/ml)				
Element	Std 1	Std 2	Std 3	Std 4	Std 5
Ca*	0.5	1.0	1.5	2.0	2.5
Cd	0.05	0.1	0.15	0.2	0.25
Cr	0.1	0.2	0.3	0.4	0.5
Cu	0.1	0.2	0.3	0.4	0.5
Fe	0.2	0.4	0.6	0.8	1.0
K	0.2	0.4	0.6	0.8	1.0
Na	0.2	0.4	0.6	0.8	1.0
Zn	0.1	0.2	0.3	0.4	0.5

* Ca standards are made in 1% La_2O_3 in 2% HNO3. Lanthinum supresses chemical interference due to sulfate and phosphate and decreases ionization interference in an air/acetylene flame.

II. **Samples:** Samples must be diluted quantitatively with 2% Ultrex HNO_3 to a concentration which falls within the standard curve designated above. The dilutions necessary are derived empirically.

For the determination of Ca, all samples must be diluted in 1% La_2O_3 as used in the standard curve.

For aspiration of sample into the flame, disposable 5 ml polypropylene tubes are convenient.

DETERMINATION

Standard parameters used for optimizing the determination of these elements are listed in Table 6.2-2. These parameters are specific to an Instrumentation Laboratories IL751 spectrophotometer employing single element hollow cathode lamps (1). Conditions may vary for other instruments.

TABLE 6.2-2. INSTRUMENT PARAMETERS FOR FLAME ATOMIC ABSORPTION ANALYSES OF METALS (IL751 SPECTROPHOTOMETER).

Metal	Wavelength (nm)	Lamp current (mA)	Flame	Bandwidth	Sensitivity (0.0044 AU)
Ca	422.7	7	lean	1	.05 ug/ml
Cd	228.8	3	lean	1	.01 ug/ml
Cr	357.9	7	rich	0.5	.06 ug/ml
Cu	324.7	5	lean	1	.03 ug/ml
Fe	248.3	10	lean	0.3	.04 ug/ml
K	766.5	7	lean	1	.01 ug/ml
Na	589.0	8	lean	0.5	.001 ug/ml
Zn	213.9	3	lean	1	.008 ug/ml

CALCULATION

Using a microprocessor based instrument, the data output is ug/ml of digested sample. A manual instrument will produce data output as Absorbance units. In this case, a linear regression analysis must be run on the standard curve in order to obtain the concentration of the element of interest in the sample. Once the concentration in ug/ml of diluted digested sample is obtained, the concentration in the original sample (oil or esters) is calculated as follows:

$$ug/g = \frac{(ug/ml - ug/ml\ blank) * dilution * total\ ml\ in\ digest}{g\ sample}$$

PRECISION

The precision of flame atomic absorption spectrophotometry determinations vary according to sample matrix. Generally ashed or acid digested samples of biological origin are precise within 10%.

NOTES

1. All glassware and plasticware must be acid washed. This can be accomplished by soaking clean glassware in a nitric acid bath containing 1:3 H_2O:concentrated HNO_3 (reagent grade). After acid-soaking, clean items should be rinsed in distilled water, dried, and stored in clean, covered cabinets.
2. Disposable pipet tips are rinsed with 2% nitric acid before use.

REFERENCES

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6.3 VAPOR GENERATION (As, Hg, Se)

INTRODUCTION

Hydride generation is utilized for the determination of As and Se in microwave HNO_3 digested oil and ester samples. The determination of Hg is done by Hg cold vapor analysis. Vapor generation methods were developed for volatile metals for which flame atomic absorption is either insensitive or unreliable. These techniques are standard methods used by the EPA (1). The drawback of hydride generation in the past has been the variability of results between analysts due to considerable technique required. The BTM Program utilizes an automated vapor generation system which makes the analyses routine and reproducible.

PRINCIPLE

In hydride generation, As and Se in acidic solution are reacted in a closed vessel for a known length of time with a known volume of a reducing agent, $NaBH_4$, to produce volatile As or Se hydrides. The hydrides are swept into a quartz cell placed in the path of the hollow cathode light beam, between the light source and the detector. A linear relationship (Beer's Law) exists between the amount of As or Se present in the cell and the amount of light absorbed at a specific wavelength.

In the mercury cold vapor technique, Hg in acidic medium is reacted with a reducing agent, $SnCl_2$, in a closed vessel for a known length of time. Elemental Hg, which is a vapor at room temperature, is produced and is swept into the quartz cell. Absorption of light at a specific wavelength is proportional to the amount of Hg present in the cell.

APPARATUS

* Atomic absorption spectrophotometer, dual beam, equipped with background correction (Instrumentation Laboratories IL751 or equivalent)
* Atomic Vapor Assembly (IL440 Thermo Jarrel Ash or equivalent)
* Strip chart recorder, equipped for remote start (Varian 9176 or equivalent)
* Fleakers, 150 ml, with lids (Fisher)
* Stir bars, teflon
* Stir plate
* Graduated cylinder, 50 ml

REAGENTS

* $NaBH_4$, ACS grade (Baxter)
* HCl, Ultrex (J.T. Baker), 1M and 3M solutions
* $SnCl_2$, ACS grade (Baxter)
* NaOH, ACS grade
* Argon
* Acetylene, 99% pure
* Compressed breathing air

PREPARATION OF STANDARDS AND SAMPLES

Arsenic: 1. **Standards.** Make 5 standards containing 10, 20, 30, 40 and 50 ng As in 50 ml 4 M HCl. Transfer entire volume of each to a 150 ml fleaker with a stir bar.

2. **Samples.** Dilute samples to a final volume of 50 ml in 4 M HCl. Transfer to 150 ml fleakers with stir bars.

Selenium: 1. **Standards.** Make 5 standards containing 10, 20, 30, 40, and 50 ng Se in 50 ml 4 M HCl. Transfer entire volume of each to a 150 ml fleaker with a stir bar.

2. **Samples.** Dilute samples to a final volume of 50 ml in 4 M HCl. Transfer to 150 ml fleakers with stir bars.

Mercury: 1. **Standards.** Make 5 standards containing 50, 100, 150, 200 and 250 ng Hg in 50 ml 1 M HCl. Transfer entire volume of each to a 150 ml fleaker with a stir bar.

2. **Samples.** Dilute samples to a final volume of 50 ml in 1 M HCl and transfer to 150 ml fleakers with stir bars.

DETERMINATION

The reducing reagents and instrument settings used for As, Se, and Hg are listed in Table 6.3-1 (2). Instrumental settings on the spectrophotometer should be optimized as described in the instrument manual. All analyses are performed with background correction. Peak height integration method is used. A four second integration is usually appropriate. However, if automatic integration is used, the absorbance curves should also be output to a strip chart recorder for verification that the true peak maximum is integrated. For As and Se, the cell is heated with a lean (blue) air/acetylene flame. For Hg, the cell is unheated.

TABLE 6.3-1. INSTRUMENT PARAMETERS FOR HYDRIDE GENERATION.

Element	Bandwidth (nm)	Wave-length	Reducing reagent	Reagent setting	Argon Flow (LPM)	Reaction time (min)
Arsenic	1	193.7	1% $NaBH_4$ in 1% NaOH	5	3-4	0.5
Selenium	1	196.0	0.3% $NaBH_4$ in 1% NaOH	5	6-7	0.5
Mercury	1	253.7	5% $SnCl_2$ in 25% HCl	5	5-6	1.0

1. **Zeroing the instrument:** Place a "blank" fleaker containing 50 ml acid (4M HCl for As and Se, 1M HCl for Hg) in the sample well of the hydride generator. Press "auto zero" on the AA and "run" on the vapor generator. The instrument will automatically flush the sample fleaker with argon, dispense the designated amount of reagent, stir the sample for a designated "reaction time" and purge the head volume again with argon, sweeping any hydrides into the quartz cell for determination of absorption. The AA will automatically "zero" on this sample.
2. **Standard curve:** A standard curve of concentration *vs* absorption is generated by analyzing the 5 concentrations of metal standard prepared above. To analyze each standard, the concentration of the standard is entered into the microprocessor on the AA as per instructions in the instrument manual, then "run" is pressed on the vapor generator. The instrument will automatically flush the fleaker with argon, dispense the designated amount of reagent, stir the sample for a designated "reaction time" and purge the head volume again with argon, sweeping any hydrides into the quartz cell for determination of absorption. Once all standards have been analyzed, the standard curve stored in the AA microprocessor is used to calculate the concentrations of metals in the samples.
3. **Samples:** Samples in 50 ml acid (4M HCl for As and Se, 1M HCl for Hg) are analyzed using the identical procedure as for the standards above.

CALCULATION

The atomic absorption spectrophotometer will calculate the concentration of metal in the sample as ppb (ng/ml) in the 50 ml diluted sample if the standards are entered as ppb. The concentration of metal per gram original sample is calculated as:

$$ng/g = C \cdot \frac{50\ ml}{V} \cdot \frac{10\ ml}{W}$$

where,

C = concentration (ng/ml) calculated by the spectrophotometer
V = volume of digested sample in 50 ml in the sample fleaker
W = the weight of the original sample in g
10 ml = total volume of the digest

PRECISION

Hydride generation and Hg cold vapor techniques are generally precise to within 10%.

REFERENCES

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6.4 ANODIC STRIPPING VOLTAMMETRY (Pb)

INTRODUCTION

Differential pulse anodic stripping voltammetry (DPASV) is used for the analysis of lead (Pb) in acid digested oils and esters. This technique was chosen because the flame AA detection limit is not sufficiently sensitive. The detection limit for Pb in the digested fish oils by anodic stripping voltammetry is about 10 ppb.

PRINCIPLE

Lead is analyzed using anodic stripping voltammetry on a Hg drop electrode (1). Briefly, Pb, in the presence of a suitable electrolyte, is deposited onto a Hg electrode which is held at a voltage potential lower than the half-wave potential of Pb. After deposition for a known length of time, the voltage at the Hg electrode is then increased in the anodic direction relative to a reference electrode, until a potential is reached which is equal to the half-wave potential for Pb. At its half-wave potential the Pb is stripped off the electrode, creating a current that is proportional to the concentration of Pb in the sample cell. The data from the analysis is the form of a voltammetric curve, where the current of Pb ions leaving the Hg electrode is represented by a peak. The method of standard addition is used to determine the concentration of Pb in the sample (2).

APPARATUS

* Polarographic analyzer, with static mercury drop electrode and automatic sample stirrer (Princeton Applied Research Model 384B or equivalent)
* Printer/Plotter (Houston DMP-40 or equivalent)
* Electrolytic reagent cleaner (Model 2014P, Environmental Sciences Associates or equivalent)
* Glass polarographic sample cells (Princeton Applied Research)
* Stir bars
* Teflon forceps
* Volumetric flask, 50 ml
* Automatic pipet, 5-ml capacity (Rainin Pipetman P5000 or equivalent)

REAGENTS

* Pb standard reference solution, 1000 ppm (Fisher)
* Sodium acetate, anhydrous ACS grade

* Acetic acid, reagent grade
* Tartaric acid, reagent grade
* Nitric acid, Ultrex grade (J.T. Baker)
* Nitrogen, ultra high purity

PREPARATION OF STANDARDS & SAMPLES

1. **Working standard:** A Pb standard solution of 1 *ug*/ml is made fresh daily by diluting the commercially prepared 1000 ppm standard with 2% Ultrex nitric acid and bringing it to volume in a 50 ml volumetric flask. This working standard is used for spiking samples for determination by the method of standard addition.
2. **Samples:** In a polarographic sample cell, 5 ml of sample is added to 5 ml of acetate buffer, pH 4.7 (Note 1) which has been electrolytically cleaned (Note 2). A stir bar is added.

DETERMINATION

Table 6.4-1 lists the instrumental parameters used in the analysis of Pb by differential pulse anodic stripping voltammetry. The method of standard addition is used for quantitation.

TABLE 6.4-1. INSTRUMENTAL PARAMETERS FOR DPASV OF LEAD.

Purge time	240 sec
Deposit time	120 sec
Initial potential	-0.8 V
Final potential	-0.2 V
Pulse height	0.2
Scan increment	2 mV/sec

1. Place a polarographic cell containing 5 ml sample, 5 ml electrolyte solution and a stir bar on the sample stirrer on the electrode. After setting up instrument parameters, press "run" and the instrument will automatically purge the sample with nitrogen, hold the mercury electrode at -0.8 for deposition of lead, then increase the potential of the mercury electrode relative to the reference electrode, in 2mV increments/sec, stopping at -0.2mV. The magnitude of the lead peak will be output to the printer/plotter in nAmps.
2. Add a volume of 1 *ug*/ml lead standard that will approximately double the peak height (this is determined empirically; however, the volume added must be small enough that the volume of the polarographic cell does not change signiificantly). Enter the concentration of Pb added (ng Pb/ml) as "standard #1".

Analyze in "standard addition" mode. Press "run". The instrument will analyze the sample as in 1 above and enter concentration and the nAmp result into a calibration curve in the microprocessor.

3. Add the same volume of 1 ug/ml lead standard again. Enter the analysis as "standard #2". Press "run". At the end of the analysis, the concentration and nAmp result will be entered into the calibration curve in the microprocessor.

4. After all standard additions are made (the Model 384B allows up to 3), press "sample" and "playback". The microprocessor will calculate the concentration of lead in the sample run initially, as ng Pb/ml, using the calibration curve established.

CALCULATION

The Princeton Applied Research 384B Polarographic Analyzer, used in this laboratory, calculates the results in ng Pb/ml in the solution in the polarographic cell.automatically. If a manual instrument is used, the standard addition calculation is done by creating a calibration curve of concentration *vs* peak height in nAmp. A linear regression is run on the curve and the y-intercept is calculated. The negative value of the y-intercept is the concentration of Pb found in the polarograpgic cell (**Figure 6.4-1**).

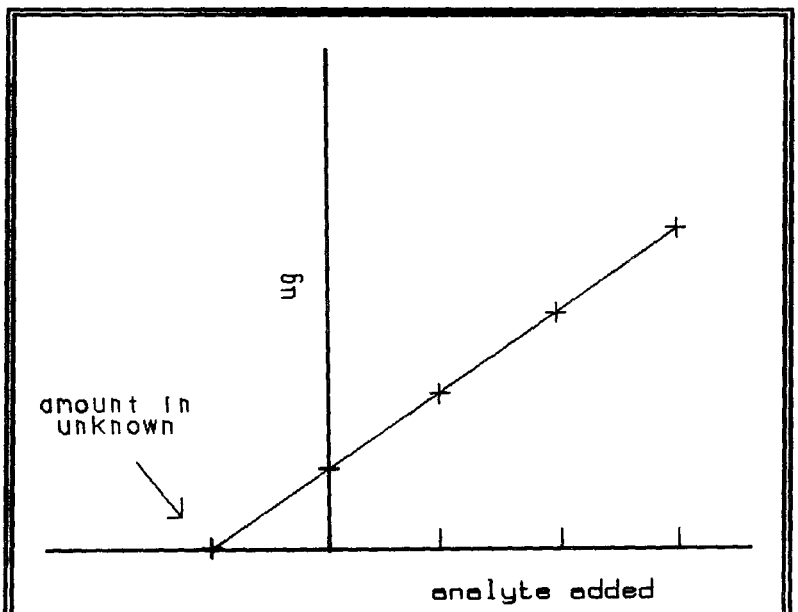

FIGURE 6.4-1. STANDARD ADDITION CALCULATION FOR Pb IN THE SAMPLE CELL.

The concentration of Pb present in the original sample is calculated as follows:

$$ug/g = S \cdot \frac{10\ ml}{X\ ml} \cdot \frac{Y\ ml}{W\ g}$$

where,

S = Pb concentration in the cell, ng/ml
X = the volume of sample digestate added to the cell
Y = the total volume of digestate prepared
W = the weight of sample digested

PRECISION

Samples analyzed in triplicate should have a relative standard deviation on less than 10%.

NOTES

1. **Acetate buffer:** Dissolve 139.45 g anhydrous sodium acetate in 300 ml water. Add 102 g glacial acetic acid and 7.5 g tartaric acid. Dilute to 1 L with water. Check pH (4.7 $\pm$ 0.1). Clean in electrolytic reagent cleaner (Note 2).

2. **Electrolysis of acetate buffer:** The cleaning system (Fig. 6.4-2) consists of a 3 L reagent cleaning flask containing a mercury pool (working electrode) at the bottom of the flask, a counter electrode, a reference electrode (Ag/AgCl), and an electronic unit (3). The mercury pool working electrode is held at a negative potential relative to the reference electrode. A platinum wire provides the electrical connection from the electronic unit to the mercury pool. A platinum wire counter electrode supplies the current required for the continuous reduction of ions from the solution onto the surface of the mercury pool.

   To remove lead from the acetate buffer prepared in Note 1, transfer it to the cleaning flask. Also fill the counter electrode sleeve with it. Fill the reference electrode with 0.1 N NaCl. These electrodes are located in a rubber stopper to the flask. Press the stopper in place, such that the counter electrode touches the mercury pool. Connect a nitrogen line to the flask to purge the system of oxygen. A gentle stream of bubbles is adequate.

   Set the dial on the instrument panel to -0.4V. Turn on the instrument. Slowly advance the voltage reading until it reaches -1.4V. Maintain these conditions in the cleaning system for 2 days - 1 week. Remove a 5 ml sample from the flask and test for presence of lead by DPASV as outlined above. When the reagent is clean, place a pipet in the solution and pump the solution out of the flask by positive displacement, achieved by turning up the

nitrogen flow into the flask (Figure 6.4-1). The buffer solution will be displaced through the pipet by the nitrogen. A length of acid-rinsed Tygon tubing can be attached to the pipet to direct the buffer to an acid-washed teflon bottle for storage.

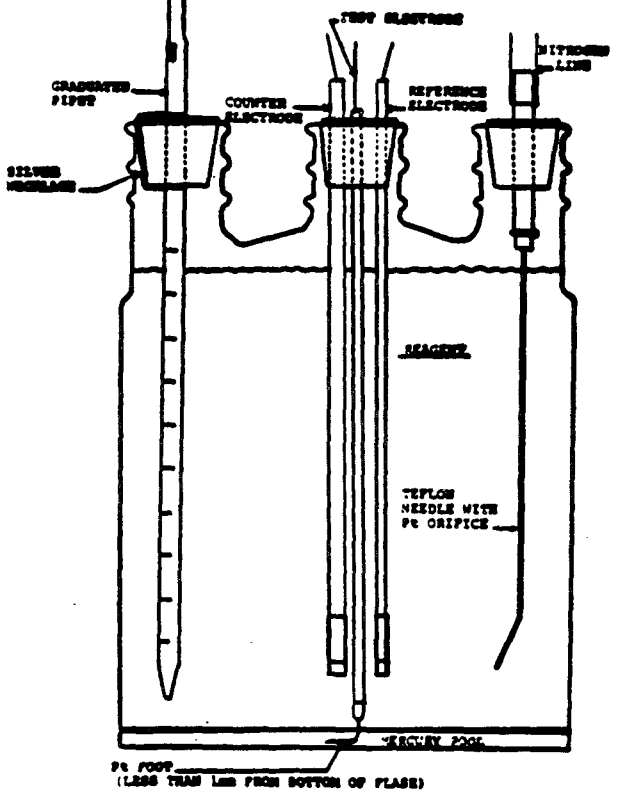

FIGURE 6.4-2. REAGENT CLEANING SYSTEM

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7 MOISTURE

INTRODUCTION

The AOAC has published a final action Karl Fischer method (#28.003-28.005) (1) for the determination of moisture in oils and esters. A collaborative study on the determination of water in oils and fats using the Karl Fischer method has been reported (2). The method employed by the BTM Program is a modification of the AOAC method for use with a Mettler DL-18 automated titrator (3,4).

PRINCIPLE

Fish oil or esters of fish oil are dissolved in methanol or chloroform-methanol and titrated with the Karl Fischer reagent to an electrometric end-point. In the titration, water is converted stoichiometrically in the presence of SO_2, methanol and a suitable base by the addition of iodine in the following reaction:

$$H_2O + I_2 + 3RN + SO_2 + CH_3CH_2OH \rightarrow (RNH)SO_4CH_3 + 2(RNH)I$$

The automated titrator uses a single component system in which the Karl Fischer reagent contains I_2, SO_2, and base. Methanol is present as a solvent. The titration is followed using a two pin platinum electrode which has a current source applied to its poles. The voltage at the electrode pins is the input signal measured. When the last traces of H_2O have been titrated the voltage drops to virtually zero which signals the electrometric endpoint.

APPARATUS

* Karl Fischer titrator (Mettler DL-18 or equivalent)
* Burette, 10 ml (Mettler or equivalent)
* Printer (Epson FX-85 or equivalent)
* Balance, 4 place, with RS232 interface (Mettler AE200 or equivalent)
* Glass titration vessel, 150 ml (Mettler or equivalent)
* Wide bore syringe (10 ml capacity)
* 1 ml syringe
* Automatic pipet

REAGENTS

* Hydranal - Composite 2 (2 mg H_2O/ml) (Reidel-deHaen)
* Methanol (ACS grade)

* Chloroform (ACS grade):methanol (1:1)
* 3M KCl/sat AgCl reference electrode solution

PREPARATION OF STANDARDS & SAMPLES

Approximately 2 g samples are used for oils and esters. Approximately 0.2 g samples are used for determination of moisture in ethanol. No sample preparation of the ethanol, oils or esters is necessary since they are freely soluble in chloroform:methanol.

DETERMINATION

TABLE 7-1. AUTOTITRATOR CONFIGURATION FOR MOISTURE ANALYSIS.

Configuration No.	Value	Explanation
1	15	switch-off delay (seconds)
2	1	report in %
3	1	pyridine-free single component KF reagent
4	0	calibration sample: water
5	1	automatic pre-titration before each run
6	9999	request stir time for each sample
7	0	no blank value
8	0	requests no sample number
9	2	acoustical signal at end of titration
A	6	max vol per titration: 6 burette strokes

1. Configure the autotitrator with the settings listed in Table 7-1.
2. Determine concentration of the Karl Fischer reagent using water as the standard: pipette 50 *u*l water into a tared titration beaker, using a 200 *u*l pipettor. The weight will automatically be entered into the microprocessor from the balance. The result is stored automatically in memory and is used in the evaluation of titrations that follow.
3. Determine the background titrant consumption due to moisture leaking into the titration beaker: using pre-titrated solvent, a 3-5 minute titration is carried out. The result is stored in the instrument as the "drift" parameter. A value of <25 *u*g/min is acceptable.
4. To determine moisture in sample: dispense 40 ml solvent into the titration vessel. The instrument will automatically titrate the solvent to dryness. Draw sample into wide bore syringe. Place the syringe on balance. Tare the balance. Inject the oil sample into the titration vessel. Reweigh the empty syringe. The weight of the oil is entered into the memory by pressing "run" (the negative sign is ignored).

5. The sample is automatically titrated to dryness and background corrections are made.

CALCULATION

Water content of the sample is calculated automatically by the autotitrator as percent moisture.

PRECISION

The measuring range of the autotitrator is 10 *ug*-500 mg water, or 1 ppm to 100%. The resolution is 1/2000th of the burette volume. Relative standard deviations are below 10% for low moisture samples and below 2% for high moisture samples.

REFERENCES

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8 SENSORY ATTRIBUTES

The BTM Program utilizes sensory analysis to determine low levels of oxidative degradation of the products and to evaluate the acceptability of BTM products for human consumption. Two sensory tests are performed on both oil and ester concentrates, odor/flavor profile evaluation and color. Odor and flavor profiles are analyzed by a trained sensory panel consisting of five to six individuals. The methods used for sensory training are presented in section 8.1. Color is analyzed by the Gardner method, using a standard scale for color. Sensory analyses have proven to be as sensitive or more sensitive than many of the instrumental methods available.

8.1 SENSORY TRAINING; TRIANGLE TESTS AND INTENSITY RANKING

INTRODUCTION

Prospective sensory panelists are trained using standard methodology (1,2). Panelists are first screened for flavor and odor sensitivity using the triangle difference test for the four basic tastes: sweet, sour, salty, and bitter. Once the panel is selected, the individuals are trained to identify specific flavor and odor components using a series of chemical standards (3,4). An unstructured scale (5) is utilized to identify weak versus strong presence of the odor and flavor components identified by the chemical standards. Finally, the responses of the individuals on the taste panel are "calibrated" to these known fish oil standards.

PRINCIPLE

The triangle difference test is a standard test used to determine that the panelists possess sufficiently sensitive taste and odor discrimination (1,2). Once selected for the panel, sensory training, utilizing chemical standards and known fish oil standards, minimizes the subjective responses of individual panelists. A consensus is reached among panelists on the definition of odor/flavor descriptors using chemical standards. Known fish oil standards are then used to reach a consensus on the intensity rating which will be assigned to a standard sample, and thus to calibrate the panelists' responses to unknown samples.

APPARATUS

* Volumetric flasks, 100 ml
* Analytical balance, capable of weighing to four decimal places
* Beakers, 40 ml
* Watch glasses to fit 40 ml beakers
* Waterbath, 50°C

REAGENTS

*	Bitter	-Caffeine, 0.035%, 0.07% 0.14%
*	Sweet	-Sucrose, 1%,2%,4%
*	Salty	-NaCl, 0.1%, 0.2%, 0.4%
*	Sour	-Citric acid, 0.035%, 0.07%, 0.14%
*	Buttery	-H and R artificial butter flavor, 0.0005%, 0.001%, 0.005%

*	Beany	-soybean oil -toasted soybeans
*	Rancid	-*cis*-4-heptenal, 5 *ug*/ml -cyclohexanecarboxylic acid, 4 *ug*/ml,200 *ug*/ml
*	Painty	-edible linseed oil heated for 4, 6, 8, and 10 days at 60°C
*	Oxidized	-soybean oil heated 10 days at 60°C -*t*-2-*t*-4-decadienal, 50 *ug*/ml -cottonseed oil heated 15 days at 60°C
*	Grassy	-*cis*-3 hexenol 500 *ug*/ml, 200 *ug*/ml -*t*-2-*cis*-6-nonadienal, 2*ug*/ml, 10*ug*/ml
*	Fishy	-trimethylamine HCl, 50 *ug*/ml -cod liver oil
*	Fruity/melon	-olive oil -esters

DETERMINATION

I. **Triangle test**

1. Prepare 100 of 500 ml of each standard at the three specified concentrations
2. Aliquot 20 ml of each of the standard solutions for the four basic flavor components (sweet, salty, sour, bitter) into 50 ml beakers.
3. Present each prospective panelist with three samples, two of which are the same and one which is different, beginning with the highest concentration.
4. The prospective panelist is required to pick the sample believed to be different.
5. Repeat this test for twelve combinations to test for each of the four basic tastes at three concentrations.
6. The prospective panelist must be correct on at least 60% of the responses in order to qualify for the panel.

II. **Training for specific odor components**

1. Provide panelists the array of standards listed above, at room temperature, clearly labeled for flavor attribute they represent.
2. Panelists familiarize themselves with the odors as they are defined by the standards provided.
3. Place a 20 ml fish oil sample in a 40 ml beaker, cover with a watch glass, and heat for not less than 10 min or more than 40 min in a 50°C water bath.

4. The panelists remove the beaker from the water bath, dry off any adhering water drops, remove the watch glass, inhale the vapors above the oil sample and identify the odor components present, using the chemical standards to define them.

5. After each panelist has identified odor components present, the panel discusses the results and reaches a concurrence on what odor components are present.

6. This "standard" fish oil sample (stored frozen at -30°C) is analyzed simultaneously with all samples analyzed for odor and flavor profile.

III. **Calibration of an unstructured scale**

1. An unstructured scale is used (Figure 8.1-1) on which total intensity of flavor, total intensity of odor and each flavor/odor component may be ranked from "absent" to "strong". The scale is 15 cm in length. In order to rank an oil, the panelist marks a slash across the scale at the point believed to best rate each attribute.

2. The "standard" fish oil is used to calibrate the scale near the "absent" end of the scale. Using a "strong" oil sample or reference oil specially created for this purpose (a thermally abused oil sample may be used), the scale is then calibrated at the "very strong" end of the scale. Each panelist is asked to mark a response on the scale and the responses of all panel members are then compared and a consensus reached on how to respond to a sample of this intensity.

3. The same samples are given in two subsequent taste panel sessions to test the calibrations of the panelists and any outlying responses are corrected.

DATE: _____

PANELIST: _____

SAMPLE: _____

SENSORY ANALYSIS OF FISH OIL

Place a vertial mark across the horizontal line according to your judgement as to the intensity of each attribute. Mark only the attributes you detect. Label each vertical line with either an "O" (odor) or "F" (flavor). The line is 15 cm. long.

	ABSENT	VERY STRONG
TIO [Total Intensity of Odor]		
TIF [Total Intensity of flavor]		
BUTTERY (sweet butter flavor)		
BEANY (soy or other raw bean flavor)		
RANCID (strong odor or flavor of rank, acrid,or oxidized fats)		
PAINTY (linseed oil or drying paint)		
OXIDIZED (sharp, stale cooking oil)		
GRASSY ("greeny", freshly cut grass, cucumbery)		
FISHY (cod-liver oil smell/flavor)		
BITTER (caffeine or quinine taste/odor)		
SWEET (pleasant, sugary, syrupy)		
FRUITY/MELON (rind of watermelon or honeydew meat)		
BURNT (carmelized scorched sugar)		
OTHER _____ (odor or flavor identified by panel member)		

FIGURE 8.1-1. ODOR/FLAVOR PROFILE SCORE SHEET.

REFERENCES

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8.2 ODOR AND FLAVOR PROFILE EVALUATION

INTRODUCTION

The odor and flavor profiles are used by the BTM Program to evaluate the fish oil and ester products destined for human consumption. In addition, the evaluation can detect low level oxidative degradation not easily detected by instrumental methods. A trained sensory panel, consisting of five to six individuals, is used to evaluate the total and individual intensities of odor and flavor of the product. The odor and flavor intensity of eleven individual flavor components are evaluated using an unstructured scale which is a modification of the Quantitative Descriptive Analysis method (QDA) (1,2).

PRINCIPLE

Fish oil or esters of fish oil (approximately 5 ml) are heated for a known length of time (10 min) in a water bath at 50°C in a beaker covered with a watch glass. Heating the sample makes volatile components more available for odor and flavor evaluation. The sensory panelists remove the beaker from the water bath, wipe off any adhering water drops, remove the watch glass, and inhale the vapors in the head space above the oil sample. Each panelist then evaluates the sample for total intensity of odor and for the intensity of eleven different odor characteristics (4-9) frequently used in the analysis of oils: buttery, beany, rancid, painty, oxidized, grassy, fishy, bitter, sweet, fruity/melon, and burnt. Intensity is recorded on an unstructured scale (15 cm, length) that ranges from "absent" to "strong" (Figure 8.1-1). The panelist marks a vertical line at a point along the unstructured scale which best reflects the intensity of the attributes detected. The panelists then take approximately 50-100 ul of sample into their mouth to analyze first for total intensity of flavor, and then for identification and quantification of intensity of the same eleven flavor components. The flavor/odor components identified have previously been defined by a consensus of the panel during the training sessions. For the purposes of this program, the following definitions were agreed upon:

TABLE 8.2-1. FLAVOR/ODOR DEFINITIONS.

BUTTERY	aroma and flavor of sweet, freshly-churned butter. A strong buttery flavor refers to a pronounced sweet butter flavor, not old or rancid.
BEANY	characteristic of soybean oil products or fresh, raw soybeans or other raw beans.
RANCID	strong, very oxidized, rank, acrid flavor and odor, very old fats.
PAINTY	resembling drying paint or strong linseed oil.
OXIDIZED	sharp; characteristic of oils exposed to air, particularly cottonseed oil. A strong oxidized flavor is termed "rancid".
GRASSY	"green" flavor or odor of freshly cut grass; slightly characteristic of cucumbers.
FISHY	characteristic odor and flavor of fish oil, such as cod-liver oil.
BITTER	taste and odor of caffeine or quinine; may be acrid.
SWEET	pleasant, sucrose odor and flavor; slightly characteristic of syrup.
FRUITY/MELON	characteristic of watermelon rind or honeydew melon; may be slightly citrus; characteristic odor and flavor of fish oil esters.
BURNT	scorched; like carmalized sugar; may be found in conjunction with sweetness.

The advantage of the unstructured scale over an interval scale is that the panelists do not have to fit their perceptions into specifically designated categories. Further, the open line scale generates data that can be analyzed by standard statistical methods which assume a normal distribution (1).

Because a panelist's response may vary between days and by time of day, each analysis is accompanied by the analysis of a standard oil which serves to calibrate their responses. In order to avoid sensory fatigue, no more than three samples are analyzed in one session. For QA purposes, each sample is analyzed by the panel in two separate sessions and the mean of the two analyses is reported.

APPARATUS

* Beakers, 40 ml
* Watch glasses
* Water bath, 50°C
* Disposable dispensing pipets
* Score sheets
* Warm water
* Paper cups

REAGENTS

*	Bitter	-Caffeine, 0.035%, 0.07% 0.14%
*	Sweet	-Sucrose, 1%,2%,4%
*	Salty	-NaCl, 0.1%, 0.2%, 0.4%
*	Sour	-Citric acid, 0.035%, 0.07%, 0.14%
*	Buttery	-Artificial butter flavor (Haarmann and Reimer), 0.0005%, 0.001%, 0.005%
*	Beany	-soybean oil -toasted soybeans
*	Rancid	-*cis*-4-heptenal, 5 *ug*/ml -cyclohexanecarboxylic acid, 4 *ug*/ml,200 *ug*/ml
*	Painty	-edible linseed oil heated for 4, 6, 8, and 10 days -at 60°C
*	Oxidized	-soybean oil heated 10 days at 60°C -*trans*-2-*trans*-4-decadienal, 50 *ug*/ml -cottonseed oil heated 15 days at 60°C
*	Grassy	-*cis*-3 hexenol 500 *ug*/ml, 200 *ug*/ml -*trans*-2-*cis*-6-nonadienal, 2*ug*/ml, 10*ug*/ml
*	Fishy	-trimethylamine HCl, 50 *ug*/ml -cod liver oil
*	Fruity/melon	-olive oil -honeydew melon

* Fish oil reference standard (Note 1)

DETERMINATION

1. Oil samples and the oil standard (5-6 ml) are placed in glass beakers, covered with watch glasses and preheated in a 50°C H_2O bath for 10 minutes prior to convening the panel. No more than 3 samples are analyzed per session due to taste and olfactory fatigue.
2. The panelist removes each beaker from the water bath, wipes off adhering water drops, removes the watch glass and inhales the vapors above the sample. Each sample is rated for total intensity of odor and for the presence the eleven odor components listed on the score sheet. Intensity is recorded on an unstructured scale (15 cm in length) that ranges from "absent" to "strong". The panelist marks a vertical line at a point along the unstructured scale which best reflects the intensity of the sample component on the score sheet.
3. Samples are then tasted in the order of increasing odor (sample with the least odor is tasted first) using disposable dispensing pipets to pick up an aliquot (50-100 *ul*) of the oil sample. The panelist takes the sample into the mouth, holds the sample on the back of the tongue, draws air into the mouth and exhales through the nose. The panelist records the flavors detected and their intensity on the score sheet.
4. The panelists rinse their mouths with warm H_2O between samples.

CALCULATION

1. The scores are calculated for each flavor component by measuring the distance along the 15 cm scale at which the panelist placed a vertical mark. The scores are recorded to the nearest 0.1 cm.
2. A mean and standard deviation are calculated for each odor/flavor component and for the total intensity of odor and flavor for each panel session. For QA purposes, an overall mean and standard deviation is calculated from all panels that evaluated a particular product.

PRECISION

The "standard" oil is evaluated at each sensory panel. During panelist training it was agreed that the standard oil would be considered to possess a value of 2.0 on the 15 cm scale. A random selection of 20 sensory panels conducted at the Charleston Laboratory yielded the following means and standard deviations for the "standard oil" *between panel sessions*: total intensity of odor, 1.68 ± 0.29 (17.5% RSD); total intensity of flavor, 1.74 ± 0.43 (24.6% RSD). The average deviations *between panelists* over the same twenty sessions was 1.68 ± 0.56 for total intensity of odor and 1.74 ± 0.75 for total intensity of flavor.

NOTES

1. A mild steam-stripped fish oil is used as a reference standard. It was decided by the panel that the reference standard would be rated at approximately 2 cm from the left end of the scale for "total intensity of odor" and "total intensity of flavor". This standard is evaluated with all samples. The samples are compared to the reference standard and rated accordingly. Tubes containing aliquots of the reference standard are stored in the freezer at -30°C and used as needed.

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8.3 COLOR DETERMINATION

8.3.1 GARDNER METHOD

INTRODUCTION

The color of fish oil and esters of fish oil is analyzed as part of the QA/QC process in the BTM Program using the standard Gardner method (1). Color can greatly affect human appeal for test products, especially as compared with placebo oils. Color can also act as an indicator of oxidative change during processing and storage.

PRINCIPLE

The color of fish oil is determined by comparison with that of a range of eighteen consecutively numbered standards of specified color, using a colorimeter (1-3). The standards used in this method are called Hellige color discs. The number of the color standard which most closely matches the sample is recorded as the Gardner number for that particular sample.

APPARATUS

* Hellige color comparator (Hellige Inc., Garden City, NY)
* Color standards discs for 1933 Gardner color scale, Nos. 620C-40 and 620C-42 (Hellige Inc., Garden City, NY)

REAGENTS

* Distilled water

PREPARATION OF STANDARDS & SAMPLES

1. The standards require no preparation.
2. The sample is determined to be clean and free of particulate matter. This may be accomplished by filtering.

DETERMINATION

1. Place 5-10 ml of sample in 12 x 115 mm glass test tube provided with the color comparator.
2. Place 5-10 ml of distilled water in the reference test tube.
3. Insert the color standard disc in the wheel of the color comparator and rotate until the color standard which most closely matches the oil sample is found.
4. Record the color standard number.

CALCULATION

The number of the color standard which most closely matches the oil sample is recorded as the Gardner Number.

REFERENCES

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9 BACTERIA

INTRODUCTION

Biomedical test materials which are encapsulated in soft gelatin for the BTM Program (steam- and vacuum-deodorized menhaden oil and ester concentrates) are analyzed for pathogenic coliform bacteria, *E. coli* and *Salmonella*. The U.S. Pharmacopeia specifies that the total bacterial count in gelatin must not exceed 1000 colony forming units per gram (CFU/g), and tests for *Salmonella* and *E. coli* must be negative (1). Bulk oils and esters are not tested for bacteria since the deodorization process kills any bacteria present in the crude oil.

9.1 COLIFORM BACTERIA

INTRODUCTION

Coliforms are determined using a presumptive multiple tube fermentation test adopted by the AOAC (2) and the FDA (3). The presumptive test is followed by confirmed tests for coliforms and *E. coli*.

PRINCIPLE

Coliform bacteria are a taxonomically heterogeneous group that are identified by the production of CO_2 from lactose within 48 hr at 35°C. The presumptive multiple tube fermentation test uses the principle of dilution to extinction to estimate the number of bacteria in the sample. Decimal dilutions of the sample are introduced into replicate tubes containing lauryl tryptose (LST) broth. The maximum dilution at which growth occurs theoretically represents the volume containing a single organism. Results are expressed as the most probable number (MPN) of bacteria per gram of sample, which is determined using an MPN probability table.

The confirmed test for total coliforms is based on the ability of coliforms to produce CO_2 in brilliant green lactose bile agar in 48 hr at 35°C. The confirmed test for *E. coli* is based on the ability of *E. coli* to grow and produce CO_2 at elevated temperature (45°C) after 24 hr.

APPARATUS

* Incubator, 35 $\pm$ 1°C
* Covered water bath, 45.5 $\pm$ 0.2°C
* Immersion type thermometer, 0-100°C
* Fleakers, 500 ml
* Sterile graduated pipets, 1 and 10 ml
* Sterile utensils for sample handling
* Glass dilution bottles with teflon lined caps
* Culture tubes, 125 mm x 15 mm

REAGENTS

* Brilliant green lactose bile broth (BGLB), 2%
* Lauryl tryptose broth (LST) (Difco), in culture tubes
* EC broth (Difco)
* Levine's eosin-methylene blue agar (L-EMB) (Difco)
* Peptone water (Difco)

* Methyl red- Voges Proskauer (MR-VP) broth (Difco)
* Koser's citrate broth (Difco)
* Plate count agar (Difco)
* Gram stain reagents (Difco)
* Methyl red indicator (Difco)

PREPARATION OF STANDARDS & SAMPLES

1. Place all equipment necessary for sample preparation under a laminar flow hood. Use aseptic technique throughout the procedure.
2. Place 10 capsules in a tared, sterile 500 ml fleaker. Weigh. Add 90 ml sterile peptone buffer and allow to stand 1-2 hr at room temperature to dissolve the gelatin capsules.
3. Label 9 LST tubes for each sample, indicating 10^1, 10^2 and 10^3 dilutions in triplicate.
4. Transfer 1 ml of the dissolved sample from the fleaker to each of the LST tubes labeled 10^1 and to a dilution bottle containing 99 ml sterile peptone buffer.
5. Transfer 0.1 ml sample from the fleaker to each of the LST tubes labeled 10^2.
6. Transfer 1 ml of sample from the dilution bottle to each of the LST tubes labeled 10^3.

DETERMINATION

I. **Presumptive Test**

1. Incubate each LST tube at 35°C for 24 hr. Check tubes for gas (i.e., displacement of gas from the fermentation vial or production of gas when tube is gently agitated). Reincubate negative tubes for another 24 hr and examine another time for production of gas.
2. Perform confirmed tests on all positive tubes (II and III below).

II. **Confirmed Test for Coliforms**

1. Transfer a loopful of LST suspension from each positive LST tube to a tube of brilliant green lactose bile broth. Incubate for 48 $\pm$ 2 hrs at 35°C. Calculate Most Probable Number (MPN) of coliforms using the MPN table, based on the proportion of confirmed gassing LST tubes for three consecutive dilutions.

III. **Confirmed Test for E. coli**

1. Transfer loopful of LST suspension from positive LST tubes in the presumptive test to a tube of EC broth. Incubate 48 $\pm$ 2 hr at 45.5 $\pm$ 0.2°C. Examine for gas production at 24 and 48 hr.
2. From each positive tube of EC broth streak a loopful of suspension to an L-EMB agar plate. Incubate 18-24 hr at 35°C. Select two colonies from the plate and transfer to PCA agar slants for morphological and biochemical tests.
3. Perform Gram stain. Examine all cultures appearing as gram negative short rods or cocci for the following biochemical activities using the API-20E test kit: indole production, Voges-Proskauer reactive compounds, methyl-red reactive compounds, use of citrate, production of gas from lactose.

CALCULATION

Results are calculated as the Most Probable Number (MPN) of coliforms or *E. coli* per gram of encapsulated material. The following table provides an example of how to record data for calculation of the MPN (3). The combination of positive tubes recorded from each dilution is then applied to a MPN probability table (Note 1).

TABLE 9.1-1. SAMPLE DATA RECORD FOR CALCULATION OF MPN COLIFORMS.

Sample	Dilution			Combination of
	10^1	10^2	10^3	Positive tubes
1	3/3	3/3	1/3	3-3-1
2	3/3	2/3	1/3	3-2-1
3	2/3	1/3	0/3	2-1-0

PRECISION

The MPN method is an estimate of the density of colony forming units (CFU) in a sample. For high density populations, the MPN is not as precise as a direct plate count. It is most precise for low levels of organisms (<10 per g). For the purposes of quality assurance of biomedical test materials, however, the presence of any *E. coli* results in rejection of the "lot" of material. The limit of detection with a 10 g sample is approximately 1 CFU/g.

NOTES

1. MPN index and 95% confidence limits for various combinations of positive results when three tubes and three dilutions are used are listed in TABLE 9.1-2 (4).

TABLE 9.1-2. MOST PROBABLE NUMBERS TABLE.

Combination of Positives	MPN index per g	95% confidence limits upper	lower
0-0-0	<0.03	<0.005	<0.09
0-0-1	0.03	<0.005	0.09
0-1-0	0.03	<0.005	0.13
0-2-0	--	--	--
1-0-0	0.04	<0.005	0.20
1-0-1	0.07	0.01	0.21
1-1-0	0.07	0.01	0.23
1-1-1	0.11	0.03	0.36
1-2-0	0.11	0.03	0.36
2-0-0	0.09	0.01	0.36
2-0-1	0.14	0.03	0.37
2-1-0	0.15	0.03	0.44
2-1-1	0.2	0.07	0.89
2-2-0	0.21	0.04	0.47
2-2-1	0.28	0.10	1.50
2-3-0	--	--	--
3-0-0	0.23	0.04	1.20
3-0-1	0.39	0.07	1.30
3-0-2	0.64	0.15	3.80
3-1-0	0.43	0.07	2.10
3-1-1	0.75	0.14	2.30
3-1-2	1.20	0.30	3.80
3-2-0	0.93	0.15	3.80
3-2-1	1.5	0.30	4.40
3-2-2	2.1	0.35	4.70
3-3-0	2.4	0.36	13.0
3-3-1	4.6	0.71	24.0
3-3-2	11.0	1.50	48.0
3-3-3	>11.0	>1.50	>48.0

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9.2 *SALMONELLA*

INTRODUCTION

An AOAC approved method employing selective enrichment and selective plating is used to detect the presence of *Salmonella* in encapsulated products (1). This is the same method used by the U.S. Food and Drug Administration (2). In the test, selective plating is followed by confirmation using a commercially available biochemical test kit. Presence of any confirmed *Salmonella* colonies in a sample test material would necessitate rejection of the sample lot for human consumption, as prescribed by the U.S. Pharmacopeia (3).

PRINCIPLE

The methodology for the isolation and identification of *Salmonella* consists of five basic steps: (1) pre-enrichment in a non-selective media, (2) selective enrichment in a media which promotes the growth of *Salmonella* while restricting proliferation of most other bacteria, (3) selective plating on brilliant green agar (BG) agar, bismuth sulfite (BS) agar and xylose lysine desoxycholate (XLD) agar, (4) identification of suspicious colonies based on colony morphology and (5) confirmed identification using biochemical tests provided in a commercial test kit (API-20E).

APPARATUS

* Incubator, 35°C
* Water bath, 48-50°C
* Sterile fleakers, 500 ml
* pH paper (pH range 6-8) with graduations of 0.4 pH units
* Sterile culture tubes, 25 ml
* Sterile culture dishes
* Sterile pipets, 1 ml with 0.01 ml graduations
* Sterile pipets, 5 and 10 ml with 0.1 ml graduations
* Innoculating loop

REAGENTS

* Lactose broth (Difco)
* Selenite cysteine broth (Difco)
* Tetrathionate broth (Difco)
* Brilliant green dye solution (Difco)
* Selenite cysteine broth (Difco)

* Brilliant Green agar (Difco)
* Bismuth sulfite agar (Difco)
* XLD agar (Difco)
* API-20E (Analytab Products Inc.)

PREPARATION OF STANDARDS & SAMPLES

1. Weigh 10 g capsules into sterile fleaker using aseptic technique.
2. Add 90 ml sterile lactose broth.
3. Cap fleaker and let stand 1-2 h at room temperature to dissolve gelatin capsules.
4. Mix by swirling and determine pH with test paper.
5. Adjust pH if necessary to 6.8 $\pm$ 0.2.
6. Incubate 24 $\pm$ 2 hr at 35°C.

DETERMINATION

1. Transfer 1 ml of incubated sample to 10 ml selenite cysteine broth and another 1 ml to 10 ml of tetrathionate broth in 25 ml culture tubes.
2. Incubate 24 $\pm$ 2 hr at 35°C.
3. Streak 3mm loopful of the incubated selenite cysteine broth and tetrathionate broth onto bismuth sulfite, XLD, and brilliant green agar.
4. Incubate plates 24 $\pm$ 2 hr at 35°C.
5. Examine plates for colonies suspected to be *Salmonella* (Note 1).
6. If bismuth sulfite agar plates have no colonies suspected to be *Salmonella*, incubate them an additional 24 hr.
7. Select 2 suspect colonies from each selective agar and innoculate into triple sugar iron (TSI) and lysine iron agar (LIA) slants (Note 2).
8. Incubate TSI slants 24 $\pm$ 2 hr at 35°C. Incubate LIA slants 48 $\pm$ 2 hr at 35°C. Caps of the tubes should be loosely in place to maintain aerobic conditions.
9. Examine slants for *Salmonella* characteristics (Note 3).
10. Apply API-20E biochemical tests to 3 presumptive TSI agar cultures which originated from selenite cysteine broth and 3 which originated from tetrathionate broth in step 3 above, using the manufacturers instructions.

CALCULATION

Presence of any confirmed *Salmonella* colonies in the sample requires retesting of the sample through the entire procedure a second time. Presence of confirmed *Salmonella* in the sample a second time requires that the analysis be carried out on another sample from that product lot. Presence of confirmed *Salmonella* in a second sample from that lot requires that the lot of product from which the samples were derived be rejected for use for human consumption.

PRECISION

This assay tests for the presence of *Salmonella,* but does not provide an estimate of bacterial concentration. Since presence of any *Salmonella* positive colonies necessitates rejection of that sample lot for human consumption, a concentration is not required. The limit of detection using a 10 g sample is approximately 1 colony forming unit per gram.

NOTES

1. Brilliant Green Agar: any blue-green, green, clear or black colonies may represent *Salmonella, Shigella, Proteus* or some *Pseudomonads* and must be subjected to biochemical screening. Therefore, transfer any colony that is not salmon-orange in color.

   Bismuth sulfite agar: any black colony with or without metallic sheen must be subjected to biochemical screening for *Salmonella*.

   XLD agar: Any red, pink, cream or black colony may represent *Salmonella, Shigella, Arizona, Providence* or *Proteus* species. Not all *Salmonella* species produce black colonies on XLD; therefore, subject any colony which is not characteristically yellow to biochemical screening.

2. Lightly touch the center of the colony with sterile innoculating needle and innoculate TSI agar by streaking slant and stabbing butt. Then, without flaming, innoculate LIA agar by stabbing butt twice and streaking slant.

3. In TSI, *Salmonella* typically produces a red slant and yellow butt, with or without blackening of the agar (H_2S production).

   In LIA, *Salmonella* typically produces a purple reaction in the butt of the tube. Most *Salmonella* cultures also produce blackening of the agar. Only distinct yellow reaction in the butt of the tube should be considered a negative reaction.

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