CHEMICAL METHODS MANUAL
Foreword

The Chemical Methods Manual was developed to provide a consolidated reference of the procedures and methods used in the Fish Inspection Laboratories of the Department of Fisheries and Oceans for the chemical analysis of fish and fish products. The methods for this manual have been selected from a number of sources and appropriate references are given. No claim is made that these methods are superior to others that may be available.

The manual is published in loose-leaf format to permit flexibility in the revision of methods and in the addition of new ones as they are published. As new methods become available, they will automatically be forwarded to holders of this manual. Comment and suggestions for improvement are welcome.

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CHAPTER 1 - CONTAMINANTS
SECTION 1: MERCURY, TOTAL

1. SCOPE AND APPLICATION

1.1 This method is applicable to fish and fish products as well as other biological tissues such as fish meal, blood, hair and sediments. The detection limit of this method is 0.01 ppm assuming a sample weight of 0.25 g.

2. PRINCIPLE OF THE METHOD

2.1 The tissue sample is digested in one of three ways:

Procedure A): oxidation with a mixture of nitric and sulfuric acids at 180 °C.

Procedure B): treatment with a mixture of nitric and sulfuric acid at 60 °C and oxidation with a solution of potassium permanganate. Titration of excess potassium permanganate with 30 % peroxide.

Procedure C): microwave digestion in the presence of nitric acid.

The oxidized mercury is converted to the elemental state with a reducing solution containing stannous chloride (or stannous sulfate), hydroxylamine sulfate and sodium chloride. The mercury is partitioned into air and determined by cold-vapor atomic absorption, or equivalent, at 253.7 nm.

3. INTERFERENCES

3.1 There are no known significant interferences.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 When preparing the sample, take into account the type of product and how it is used and prepared by the consumer. Store so as to ensure product integrity.
5.1.1 For products that are packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained. Analyze the samples individually.

5.1.2 For products that are packed in a medium that may be or is normally used by the consumer, e.g., fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained. Analyze the samples individually.

5.1.3 Individual whole fish.

5.1.3.1 For a composite sample, remove the entire fillet from each fish. Grind or blend all the fillets together such that a single homogenized sample is produced.

5.1.3.2 For individual samples, first record the weight of each fish. Remove a portion from each fish and analyze the samples for mercury individually.

5.1.4 Fillets.

5.1.4.1 For a composite sample, grind and/or blend all the fillets into a single homogenized sample.

5.1.4.2 For individual samples, see section 5.1.3.2.

6. APPARATUS

NOTE: Unless specifically mentioned, apparatus pertains to all three digestion procedures.

6.1 Cold-vapor atomic-absorption system, or equivalent, equipped with a mercury vapor lamp, auto-sampler, proportioning pump, reagent manifold and mixing coils (see figure 1).

6.2 Digestion apparatus:

6.2.1 Procedure A: aluminum hot block capable of heating and maintaining samples in 50 mL tubes at 180 °C.

6.2.2 Procedure B: agitating water bath capable of heating and
maintaining samples in 50 mL tubes at 60 °C.

6.2.3 **Procedure C**: Microwave digestor with Teflon digestion vessels.

6.3 Tissue homogenizer, blender, or food grinder.

6.4 Glass "debubbler" tube, for separating the gas and liquid phases prior to atomic absorption measurement (see Figure 2).

6.5 Vortex mixer.

6.7 Strip chart recorder, integrator or computer equipped with software capable of collecting and processing data.

6.8 50 mL graduated Taylor tubes or equivalent.

7. **REAGENTS**

NOTE: Unless specifically mentioned, reagents pertain to all three digestion procedures.

7.1 Concentrated nitric acid (HNO₃), reagent ACS.

7.2 Concentrated sulphuric acid (H₂SO₄), reagent ACS.

7.3 Sodium Chloride (NaCl), certified.

7.3.1 Sodium Chloride solution (20 % - w/v ) - add 200 g of sodium chloride to a 1000 mL volumetric flask. Add approximately 500 mL of distilled water and stir for 30 minutes. Bring to the 1000 mL mark with distilled water.

7.4 Purified stannous sulphate (SnSO₄) or stannous chloride (SnCl₂).

7.5 Certified hydroxylamine sulphate.

7.6 Reducing solution. To 3000 mL of distilled water in a 4000 mL volumetric flask add the following, stirring well after each addition:
   a) 400 mL concentrated H₂SO₄;
   b) 120 mL of 20 % NaCl;
   c) 40 g of hydroxylamine sulfate;
   d) 80 g of stannous sulfate (SnSO₄) or 84 g of stannous chloride (SnCl₂).
After the final addition, stir for approximately 30 min. Bring to the 4000 mL mark with distilled water and mix thoroughly.

7.7 Certified potassium dichromate (K₂Cr₂O₇).

7.7.1 5\% (w/v) potassium dichromate solution made up with distilled water.

7.8 **Digestion procedure A and B:** Digestion acid: combine 500 mL of concentrated HNO₃ with 2000 mL concentrated H₂SO₄. Stir well and cool before using.

7.9 **Digestion procedure B:** potassium permanganate (KMnO₄).

7.9.1 **Digestion procedure B:** 6\% solution (w/v) of potassium permanganate (KMnO₄) made up with distilled water.

7.10 **Digestion procedure B:** Hydrogen peroxide (H₂O₂, 30\%).

7.11 Rinse all glassware with a 1 + 3 mixture of concentrated HNO₃ and distilled water.

7.12 1000 ppm mercury standard.

7.12.1 1000 ppm certified reference mercury standard.

7.12.2 Alternatively, prepare a 1000 ppm primary standard by dissolving 0.1354 g of HgCl₂ in approximately 75 mL of distilled water, add 5 drops of concentrated H₂SO₄ and transfer to a 100 mL volumetric flask. Dilute to the mark with distilled water. Solution is stable for approximately 1 month.

7.13 1 ppm working standard - add 1 mL of 1000 ppm certified reference standard or 1000 ppm primary standard into a 1000 mL volumetric flask. Add 10 mL of concentrated sulfuric acid and 10 mL of 5\% potassium dichromate. Dilute to the mark with distilled water.

**8. PROCEDURE**

8.1 Sample digestion.

8.1.1 **Digestion procedure A**

8.1.1.1 Accurately weigh between 0.15 and 0.50 g of sample into a
50 mL graduated Taylor tube (allow the sample to partially thaw prior to weighing). Sample weights will depend on the difficulty of digestion, e.g., fish meals, fish protein concentrates and fish oils will necessitate smaller sample weights.

8.1.1.2 Prepare 5 standards by adding 0, 50, 100, 200 and 300 µL of the 1 ppm mercury working standard to 5 separate 50 mL graduated Taylor tubes. This represents 0, 50, 100, 200 and 300 ng of total mercury. Both standards and blank are taken through the entire procedure.

8.1.1.3 Add 5 mL of digestion acid to the digestion vessel containing the weighed sample, standards and blank. Ensure that the acid covers the entire sample. For high fat samples such as fish liver, add an additional 2 or 3 mL of fuming nitric acid following the addition of the digestion acid, to ensure complete sample destruction.

8.1.1.4 Place tubes on the aluminum heating block and bring to 180 °C. Maintain at this temperature for 12 hours. At this point, the digestate should be clear (see Appendix A). If there is evidence of charring, the sample is unrecoverable and must be prepared again.

8.1.1.5 When digestion is complete, remove the sample from the block and cool to room temperature. Cooling may be accelerated by placing the vessels in a bath of cool water.

8.1.1.6 Add approximately 21 mL of distilled water to each tube. Allow the samples to cool to room temperature, adjust the final volume to 25 mL mark using distilled water and vortex.

8.1.2 Digestion procedure B

8.1.2.1 Follow sections 8.1.1.1, 8.1.1.2, 8.1.1.3.

8.1.2.2 Loosely cap the digestion vessel and place in an agitating water bath set at 60 °C for 2 hours or until digestion is complete (see Appendix A). The cap should permit the escape of nitrous oxides.

8.1.2.3 At this point, the digest should be clear (see Appendix A). If there is evidence of charring, the sample is unrecoverable and must be prepared again.
8.1.2.4 Follow section 8.1.1.5.

8.1.2.5 Slowly add 15 mL of 6 % KMnO₄ with continuous mixing in an ice bath. Allow to stand for a minimum of two hours, or preferably overnight.

8.1.2.6 Titrate the sample solution with 30 % H₂O₂; add the H₂O₂ dropwise with continuous mixing on a vortex mixer until the solution just clears. Avoid addition of excess H₂O₂. If necessary, titrate any excess H₂O₂ with a weak KMnO₄ solution to a very pale pink color.

8.1.2.7 Follow section 8.1.1.6.

8.1.3 Digestion procedure C

8.1.3.1 Accurately weigh duplicate 1 g samples into the Teflon digestion vessels. Standards, blanks and check samples are also prepared and digested in the same way.

8.1.3.2 Add 5 mL of concentrated HNO₃. Cap the vessels, close the vents and place in the carousel.

8.1.3.3 Place the carousel into the microwave digestor as per manufacturer’s instructions.

Run the microwave gradient program:

<table>
<thead>
<tr>
<th>Power (%)</th>
<th>Time (min)</th>
<th>Max Pressure (PSI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>5</td>
<td>20</td>
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<tr>
<td>40</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>85</td>
<td>15</td>
<td>120</td>
</tr>
</tbody>
</table>

8.1.3.4 After the digestion is complete, leave the tubes in the carousel for about 10 minutes to allow the samples to cool and the pressure to decrease.

8.1.3.5 Vent the vessels slowly.

8.1.3.6 Quantitatively transfer the contents of the vessels to calibrated Folin-Wu tubes or 50 mL volumetric flasks. Allow the solutions to cool and make to volume (50 mL) with deionized water.
8.2 Set up the cold-vapor atomic-absorption system, or equivalent, according to the manufacturer’s instructions.

8.3 Activate the equipment 30 minutes prior to analysis.

8.4 Adjust the "zero" on the recorder and the cold-vapor atomic-absorption system, or equivalent, with the reducing solution passing through the system.

8.5 Place the sample, standards and blank, and check samples on the auto-sampler turret and turn the auto-sampler to on.

8.6 Set up the autosampler and reducing solution pump such that the sample is drawn into the tubing, mixed with reducing solution and air, and passed through a glass mixing coil. The resulting vapor is passed over a sulfuric acid trap and into the mercury monitor. The rate of reducing solution and air addition and their ratio is such that the resulting peaks yield a standard curve that is linear over the range described by the standards.

8.7 Output from the detector should be to a device from which peak areas or peak heights may be measured.

9. CALCULATIONS

9.1 Prepare a calibration curve of peak height or peak area versus nanograms Hg in the standards.

9.2 Determine the mercury concentration in the sample by comparing the sample peak height or peak area to the calibration curve, taking into account the sample weight. Express the result in terms of total mercury on a wet weight basis (ppm).

10. PRECISION AND ACCURACY

10.1 The standard deviation of triplicate analyses has been shown to be 0.04 at 0.10 ppm and 0.05 at 0.5 ppm Hg, or better. The recovery of mercury from spiked samples is typically 95 % or better.

11. REMARKS

11.1 Oxides of nitrogen will absorb at 253.7 nm. It is for this reason that hydroxylamine sulphate is added to the reducing solution.
11.2 Microwave digestion increases the overall sensitivity of the system because of the larger sample size that can be accommodated.

11.3 Vent the microwave digestion vessels in the fume hood as acid vapors may be quite injurious to the analyst.

11.4 Microwave digestions can be completed in less than 45 minutes from start to finish.

12. REFERENCES


APPENDIX A

Methods for Verifying Whether Sample Digestion is Complete

Note: This applies to all three digestion procedures

To ensure accuracy and consistent results, it is essential that digestion be complete, especially for very fatty samples such as eel, herring and fish oils. The following are three techniques for verifying that sample digestion is complete:

1) Perform a triplicate analysis for each of three different sample weights (e.g., 0.1, 0.2 and 0.4 g). Allow the samples to digest for the same length of time. All nine results should be comparable if digestion is complete for the largest sample weights. If the results are different, the digestion period must be extended or sample size reduced. If digestion appears to be complete, then the sample size used in the future for similar material should be in the middle of the range used in the test (e.g., between 0.2 and 0.3 g).

2) At the end of the normal digestion time, add a few drops of fuming nitric acid down the inside wall of the digestion vessel. Digestion is incomplete if charring occurs upon contact of the two liquids.

3) Examine the sample digest under light at the end of the normal digestion time. Allow a narrow beam of white light to pass through the sample digest at right angles to the axis of the container.
   a) If the light beam is dispersed when viewed from the top of the container, digestion is probably incomplete.
   b) If the light beam goes through the solution with more or less no dispersion, digestion is probably complete.

Safety Note:

Wear safety goggles and wipe digestion vessels dry prior to attempting method No. 3 as described above. Drops of water hitting a hot light bulb could cause it to shatter.
Figure 1: Analysis Manifold
Figure 2: Debubbler
CHAPTER 1 - CONTAMINANTS
SECTION 2: TOTAL, INORGANIC AND ORGANIC MERCURY

1. SCOPE AND APPLICATION

1.1 This method is applicable to the determination of total, inorganic and organic mercury in fish and fish products as well as other biological tissues. The detection limit of this method is 0.002ppm.

2. PRINCIPLE OF THE METHOD

2.1 The tissue sample is digested at 100°C using a 45% NaOH solution containing cysteine as a mercury binder.

2.2 Inorganic mercury analysis: After binding to cysteine in solution, inorganic mercury (Hg²⁺) is reduced to elemental mercury by stannous ions in a strongly basic solution. The organic mercury-cysteine complexes are not reduced in this process.

2.3 Total mercury analysis: In another subsample, a large excess of cadmium ion (Cd²⁺) is added with the stannous ions (Sn²⁺) to displace all type of mercury from cysteine in a strongly basic solution, and all forms of mercury are reduced to elemental mercury by stannous ions to give a total mercury determination. The result thus obtained is subtracted from inorganic mercury result, as determined in section 2.2, to give organic mercury by difference.

2.4 Equations:

\[
\begin{align*}
\text{CH}_3\text{Hg} + \text{cysteine OH}^- , \text{Cl}^- & \rightarrow \text{Hg} + \text{Sn}^{+2} \\
\text{Hg(cysteine)}_2 \text{OH}^- , \text{Cl}^- & \rightarrow \text{Hg} + \text{Sn}^{+4} \\
\text{CH}_3\text{Hg} + \text{cysteine OH}^- , \text{Cl}^- & \rightarrow \text{Hg} + \text{Sn}^{+4} + \text{Cd(cysteine)}_2 \\
\end{align*}
\]
Hg (cysteine)$_2$ \( \frac{\text{OH}^-, \text{Cl}^-}{\text{Sn}^{2+}, \text{Cd}^{2+}} \) Hg + Sn$^4+$Cd(Cysteine)$_2$

2.5 The elemental mercury is portionned into air and determined either by flameless atomic absorption at 253.7 nm or by a mercury monitor.

3. INTERFERENCES

3.1 There are no known significant interferences.

4. SAMPLING AND STORAGE

4.1 Commercial shipment: Take a representative sample from the product lot and store as to maintain sample integrity.

4.2 Survey samples: Fish may be either pooled or individual. For species normally greater than 30 cm in length, an individual fish may be used as a sample. For species less than 30 cm in length, a pooled sample is required. Store as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Commercial shipment: Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For fish and fish products that contains no free liquid, comminute the sample until homogeneous.

5.1.2 For products that are packed in water, brine or similar medium that is normally discarded by the consumer; open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

5.1.3 For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil; transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.2 Survey samples:

5.2.1 For individual fish, weigh and measure the fork-length, i.e. from the nose to the fork of the tail, for size correlation.
5.2.2 For a pooled sample, determine the average values for length and weight of the fish.

5.2.3 Pass the skinned fillets through a commercial meat grinder a sufficient number of times to obtain a homogeneous blend (e.g. three times).

5.3 Collect the homogenized sample into a thoroughly cleaned, sealable plastic pot or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. APPARATUS

6.1 Flameless atomic absorption spectrophotometer with a mercury hollow cathode vapour lamp or a mercury monitor.

6.2 Multimeter set on mv scale.

6.3 Chart recorder (optional).

6.4 Heating stirring hot plate.

6.5 Taylor graduated digestion tubes.

6.6 Aluminium block with bored holes to fit the Taylor digestion tubes.

6.7 Automatic pipettes 1000 uL.

6.8 Vacuum pump.

6.9 Gas-air manometer.

6.10 Tygon tubing ½" diameter (I.D.).

6.11 Hot-plate thermometer.

6.12 Universal bottle top dispenser
   2 of 1-10 mL
   1 of 1-50 mL

6.13 Reagent containers 1 or 2 L in plastic or glass.
6.14 Reaction vessel (see fig. I) with dreshsel head having inlet converted to a glass funnel. Alternatively, a rubber stopper can be used.

6.15 Midget impinger.

6.16 Charcoal filter trap.

7. REAGENTS

7.1 Sodium chloride (NaCl).

7.1.1 Sodium chloride solution (1%).

7.2 Sulfuric acid (H\textsubscript{2}SO\textsubscript{4}).

7.3 4.5N H\textsubscript{2}SO\textsubscript{4} - 1% NaCl. To 35 g of NaCl add 3000 mL of H\textsubscript{2}O and 441 mL of H\textsubscript{2}SO\textsubscript{4}. Dilute to 3500 mL.

7.4 L-Cysteine.

7.4.1 L-Cysteine solution (1%)

7.5 Stannous chloride (SnCl\textsubscript{2}).

7.6 Stannous chloride solution. Add 10 g of SnCl\textsubscript{2} and 1 g of L-Cysteine. Dilute to 500 ml with 4.5N H\textsubscript{2}SO\textsubscript{4} - 1% NaCl.

7.7 Cadmium chloride (CdCl\textsubscript{2}).

7.8 Stannous chloride-Cadmium chloride solution. Heat just to boil a solution of 10g of SnCl\textsubscript{2}, 2.5g of CdCl\textsubscript{2}, and 1g of L-Cysteine. Cool and dilute to 500 mL with 4.5NH\textsubscript{2}SO\textsubscript{4} - 1% NaCl Hydroxide.

7.9 Sodium hydroxide (NaOH).

7.9.1 Sodium Hydroxide solution (35% and 45% ).

7.10 n-Octanol.

7.11 Ethanol (95%)

7.12 Antifoam solution. Add 1 mL of n-octanol to 9 mL of Ethanol (95%)

7.13 Mercury chloride (HgCl\textsubscript{2}).
7.13.1 Mercury primary standard (1000 ug/mL). Dissolve 0.1345 g HgCl₂ in ca 75 mL distilled water, add 5 drops conc H₂SO₄, and dilute to 100 mL. Solution is stable for ca 1 month at room temperature. Alternatively a commercially prepared primary standard may be used. If stored in a polyethylene container in a refrigerator, the primary standard is stable for up to a year.

7.13.2 Mercury working standard (10 ug/mL). Dilute 1 mL of mercury primary standard in 100 mL of H₂O.

7.14 Methyl mercury chloride (CH₃HgCl).

7.14.1 Methyl mercury primary standard (500 ug/mL). Dissolve 62.58 mg of CH₃HgCl in 100 mL of water.

7.14.2 Methylmercury working standard (5 ug/mL). Dilute 1 mL of the primary standard in 100 mL of H₂O.

8. PROCEDURE

8.1 Accurately weigh between 0.1 and 0.5g of homogenized sample in a 50 mL graduated Taylor digestion tube. (Allow the sample to partially thaw prior to weighing). Sample weights will depend on the difficulty of digestion; e.g. fish meals, fish protein concentrate and fish oils will necessitate smaller sample weights.

8.2 Prepare daily standards containing 0.1 to 0.4 ug Hg (10, 20, 30 and 40 uL of working mercury chloride solution) or 0.2 ug Hg (40 uL of methyl mercury working standard) to graduated Taylor digestion tube, and take both standard and blank through the entire procedure.

8.3 Set up the flameless atomic absorption spectrophotometer or Mercury Monitor according to the manufacturer's instructions with the mercury lamp. Adjust the absorption cell in place if necessary. Start vacuum pump. Adjust vacuum for 1 liter/min. Adjust the "zero" on the spectrophotometer or mercury Monitor and the recorder.

8.4 Total Mercury Analysis

8.4.1 To the Taylor graduated digestion tubes containing the samples, standards (0.1 to 0.4 ug Hg) and blanks add 1.0 mL of cysteine solution (1%), 5.0 mL 1% NaCl, and 3.0 mL 45% NaOH.

8.4.2 Digest 1 hr at 100°C while continuously shaking the tubes.
8.4.3 Cool and dilute to 50 mL.

8.4.4 Transfer 1.0 mL to the reaction vessel with three drops of antifoam solution, 5.0 mL SnCl₂ - CdCl₂ solution and 5.0 mL of 35% NaOH. Immediately record the deflection. (See fig. 2C).

8.5 Inorganic Mercury Analysis

8.5.1 Proceed as in 8.4 but use methyl mercury as standard (0.2 ug Hg). Replace SnCl₂ - CdCl₂ solution with SnCl₂ solution in step 8.4.4. Record deflection. (See fig. 2D).

9. CALCULATIONS

9.1 Prepare a calibration curve of peak height versus ug Hg in the standards.

9.2 Determine the mercury concentration in the intralaboratory check sample (if necessary), and in the sample by comparing the sample peak height to the calibration curve taking into account the sample weight and the dilution factor. Express result in terms of total mercury or inorganic mercury on a wet weight basis (ppm). Subtract inorganic mercury from total mercury to obtain organic mercury content in samples.

\[
\text{ppm (ug/g)} = \frac{\text{height sample (cm)}}{\text{weight sample (g)}} \times \frac{\text{height calibration (cm)}}{\text{ug}} \times \frac{\text{dilution factor (if necessary)}}{1000}
\]

10. PRECISION AND ACCURACY

10.1 A freeze-dried fish sample, Standard Reference Material No. 50, albacore tuna, and a bovine liver sample, Standard Reference Material No. 1577, are available from the Office of Standard Reference Materials, National Bureau of Standards, Washington, D.C. 20234. These or similar intralaboratory tuna check samples are analyzed routinely every run. The calculated precision is 11.0%.

10.2 Triplicate analyses are done for both total and inorganic mercury.

10.3 The detection limit of this method is approximately 0.002 ppm for total and inorganic mercury.
10.4 The recovery of mercury from spiked samples was 99.8\% for total mercury and 107.2\% for inorganic mercury. Not more than 7\% of organic mercury was found in the inorganic mercury analysis for spiked samples containing up to 100 ppm methyl mercury.

11. REFERENCES


Schematic representation of the assembled apparatus

Fig. 1

Representation of a actual recording of a) blank, b) calibration curve, c) total mercury, d) inorganic mercury.

Fig. 2
CHAPTER 1 - CONTAMINANTS
SECTION 3: ORGANOCHLORIDES, PESTICIDES, PCBs, AND PCB CONGENERS

1. SCOPE AND APPLICATION

1.1 This method is applicable to fish, fish by-products, marine mammals and marine mammal by-products for the quantification of total PCBs, PCB congeners and Organochlorine Pesticides (OCs).

2. PRINCIPLE OF METHOD

2.1 Compounds are extracted from the sample with ethyl acetate. Lipid co-extractives are partially removed by Gel-permeation chromatography (GPC). Clean-up and separation are achieved by absorption chromatography on deactivated Florisil. Final separation and quantification are done by Glass-Liquid Chromatography/Electron-Capture Detection (GLC/ECD). Compounds of interest are listed in Annex A.

3. INTERFERENCES

3.1 Unidentified compounds have been observed which create negative peaks at various retention times up to p,p'-DDE. A late-eluting negative peak may lengthen the GLC time required to elute the pesticide fraction.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Commercial shipments. Take a pooled sample representative of the lot, consisting of 5 sub-samples, with a combined minimum weight of 4 kg of round fish or 2 kg of fillets.

4.1.1 Survey samples. Fish may be either pooled or individual. For species normally greater than 30 cm in length, an individual fish may be used as a sample. For species less than 30 cm in length, a pooled sample is required.

4.2 Sample may be shipped from the field in a plastic bag if desired. (Note: All plastic materials in contact with the sample at any stage must be checked for possible interfering substances.)

4.3 Store the sample so as to maintain sample integrity.
5. **SAMPLE PREPARATION**

5.1 It is important to take into account the type of product and the way it is used and prepared by the consumer.

5.1.1 For products stored in water, brine or a similar medium that is usually discarded by the consumer, open the package and drain the product over a sieve of an appropriate size for 1 to 1½ minutes. Grind the part of the sample retained by the sieve until a homogeneous mixture is obtained. If the texture of the sample is too hard to be homogenized, pass the sample through a grinder a sufficient number of times to obtain a uniform mixture. The use of a food mill may be sufficient if the sample is large.

5.1.2 For products that do not contain free liquid, thaw in the package (if the product is frozen). Pass the sample through a grinder a sufficient number of times to obtain a uniform mixture.

5.1.3 For frozen frosted flesh: defrost and discard the melt water and prepare the sample as described in 5.2.

5.1.4 For frozen products with shell or carapace: remove the head, shell or carapace. Prepare the sample as described in 5.2.

5.1.5 For dried products: prepare the sample as described in 5.2.

5.1.6 For breaded products: remove breading before homogenization.

5.1.7 For fish eggs and caviar, manually stir until homogeneous.

5.2 Grind the sample until homogeneous. Place the homogenate in a plastic or glass container washed with diluted nitric acid. Store in the freezer until use. Ensure that the sample is homogeneous at the time of weighing. If a supernatant is present, re-homogenize the sample before use.

6. **APPARATUS**

6.1 Gas-liquid chromatograph equipped with split/splitless injector and with an electron-capture detector (GLC/ECD).

6.2 Capillary-fused borosilicate glass columns,
6.3 Homogenizer, food processor or food blender.

6.4 Gel-permeation chromatograph (GPC) with a 600 mm glass column, filled with 60 g of BIO-Bead SX-3 100/200 mesh.

6.5 Rotary Evaporator or similar.

6.6 Filter column for the workup described in 8.4: 30 mL fritted-glass vacuum filter of medium porosity (see Annex B).

6.7 Chromatographic preparative glass columns: 450 mm X 10 mm i.d. with a fitted reservoir of 60 mm X 60 mm i.d. (see Annex B).

7. MATERIALS AND REAGENTS

7.1 Distilled-in-glass solvents. Solvents should be suitable for use in pesticide residue analysis, free of substances which interfere with ECD. Solvents should be tested by concentrating 1/250 and injecting 1 µL in the GLC/ECD.

7.1.1 Hexane, C₆H₁₄.

7.1.2 Ethyl Acetate, C₆H₄O₂.

7.1.3 Methylene Chloride, CH₂Cl₂.

7.1.4 Acetonitrile, C₂H₃N.

7.2 Chromatographic eluants

7.2.1 Eluant I: C₆H₁₄.

7.2.2 Eluant II: CH₂Cl₂/C₂H₃N/C₆H₁₄/(49.65:0.35:50.0) (v/v/v).

7.3 Sodium Sulfate, anhydrous, granular, Na₂SO₄.

7.3.1 Purification of Na₂SO₄: heat at 600 °C for 16 hrs. or overnight.

7.4 Florisil PR Grade 60/100 mesh.

7.4.1 Storage of florisil: remove from shipping containers and store in dark-capped or stoppered bottles.
7.4.2 Deactivation of Florisil: heat the Florisil at 130 °C for 16 hrs to burn off possible phthalate residues. (Florisil can be stored for long periods at room temperature in closed amber glass containers.) Heat at 130 °C in open containers for at least 16 hrs prior to deactivation with water. Deactivate with 2 % water (wt/wt). Place in small container, i.e., 4 L amber glass bottles. Gently mix by tumbling action at room temperature for 1 hr. Let stand overnight, then prior to use, gently retumble for ½ hr at room temperature. This deactivation procedure is required to homogenize the florisoril because the amount of deactivation may vary from batch to batch. The variation is from ½ % by weight of water to 2½ %. The elution pattern should be checked for each batch. If any adjustment is required, correct the elution amounts to obtain the expected separation for the PCB/pesticide split. The prepared florisoril is kept in closed amber glass containers (see also 8.10, 8.11 and 8.12).

7.5 Primary standards

Obtain certified commercial custom standards mixture or obtain certified individual standards of all compounds of interest. Many suppliers offer certified standards, (e.g., Ultra Scientific, cat. # US-127, HSS-261 and RPC-EPA-1), or they can make and certify any mixture required.

7.5.1. Working standards

Dilute commercial certified standards mixture from 1000 μg/mL to working levels ~10-30 ng/mL with hexane and ~250 μg/mL for Aroclor 1254.

7.6 Bio-Beads SX-3 100/200 or similar.

8. PROCEDURE

8.1 Weigh 5 g of sample homogenate (or 1 g for high-fat sample) into a 25 X 150 mm test tube. Add approximately 5 g anhydrous Na₂SO₄ and 20 mL C₆H₈O₂.

8.2 Blend with the high-speed homogenizer for 1 minute.

8.3 Prepare a filter column (see 6.7) with approximately 5 g anhydrous Na₂SO₄. Use a 250 mL Round Bottom Flask (RBF) as a receiver.
8.4 Add the contents of the test tube to the filter column. Filter by suction.

8.5 Rinse the test tube with approximately 10 mL C₆H₈O₂. Repeat this step 3 times. Pour the rinses through the filter column.

8.6 Evaporate the solvent to almost dryness (~½ mL) on a rotary evaporator. Keep the water-bath temperature below 35 °C. If some water remains, add 50 mL of C₆H₈O₂ and re-evaporate. Repeat if necessary. Transfer quantitatively to a 10 mL volumetric flask and add ethyl acetate to the 10 mL mark. For fatty or difficult samples, use a 25 mL volumetric flask instead of the 10 mL volumetric flask.

8.7 Load a 5 mL aliquot of the sample onto the GPC by pushing 8 mL with a 10 mL Luer-type lock syringe into the 5 mL GPC loop. Elute with ethyl acetate, collecting the 96-211 mL fraction¹ in a 250 mL flat-bottom flask.

¹ The actual collect fraction, in mL, is determined for each GPC column by an elution pattern done on a spike sample.

8.8 Add 20 mL hexane to the GPC eluant collected and evaporate to 1-2 mL. Add another 20 mL hexane and evaporate to almost dryness. Add hexane to bring the volume of the extract to 4 mL.

8.9 Prepare a 10 x 450 mm chromatography column (Annex B, Figure 1): add 300 mm of deactivated Florisil and top with 20 mm of anhydrous Na₂SO₄. Tap the column to settle and compact the Florisil.

8.10 Elute Fraction I: C₆H₁₄

8.10.1 Add the extract to the column. Rinse the flask with 4 mL of C₆H₁₄ and immediately add the rinse to the column. Repeat this last step 2 more times. Do not allow the liquid level to fall below the top of the sodium sulfate. Once the last rinse is almost at the top of the sodium sulphate level, elute with 27 mL of C₆H₁₄. The precise volume of eluate should be determined by an elution pattern based on a recovery test. Collect eluate in a 250 mL RBF. Evaporate on a rotary evaporator to about 10 mL. Transfer in a 25 mL volumetric flask and add hexane to the 25 mL mark.
8.11 Elute Fraction II: CH₂Cl₂/C₂H₆N/C₂H₄/(49.65:0.35:50.0) (v/v/v).

8.11.1 Add 65 mL of Eluant II to the column. Collect in a 250 mL RBF. The precise volume of eluate should be determined by an elution pattern based on a recovery test. Evaporate on a rotary evaporator to almost dryness. Transfer to a 25 mL volumetric flask and add hexane to the 25 mL mark.

8.12 GLC/ECD, capillary column chromatography

8.12.1 The following are typical operating conditions and recommended columns:

8.12.2 Injector temperature: 220 °C

8.12.3 Detector temperature: 300 °C (Ni⁶³)

8.12.4 Oven temperature ramp: 120 °C for 1 min.; 15 °C/min. to 165 °C; hold 1 min.; 1 °C/min. to 185 °C; hold 1 min; 2 °C/min. to 220 °C; hold 20 min.

8.12.5 Column: Hewlett-Packard Ultra-1 (100 % phenyl silicone), 30 m X 0.25 mm I.D. X 0.25 μm.

8.12.6 Carrier gas: He at 25 psi (pound per sq. inch).

8.12.7 Make-up gas: 5 % Me/Ar, 60 mL/min.

8.13 Alternate GLC/ECD separation

8.13.1 Injector Temperature: 250 °C.

8.13.2 Detector Temperature: 300 °C (Ni⁶³).

8.13.3 Columns: (1) 30 m Megabore DB-1 or DB-5 for Total PCB and OC; or (2) 30 m x .32 mm DB-1 or DB-5 for PCB congeners and OC. Note: the Megabore column provides adequate resolution and faster run time.

Oven Temperature:
Column (1) - Typical programming: Initial 140 °C; Ramp 1--20 °C/min to 180 °C; Ramp 2--1 °C/min to 190 °C; Ramp 3--5 °C/min to 220 °C; Hold 5 min.

Column (2) - Typical programming: Initial 140 °C; Ramp 1--20 °C/min to 200 °C; Ramp 2--1 °C/min to 220 °C, Ramp 3--
5 °C/min to 250 °C; Hold 20 min.

8.13.4 Carrier Gas: He at appropriate flowrate.

8.13.5 Makeup Gas: 25-35 mL/min, 5 % Me/Ar.

9. **CALCULATION**

9.1 Peak areas of compounds are measured and calculated against standards by ESTD (external standard method).

9.2 Total PCBs are defined as the sum of the 8 peaks listed in Annex C, Table 1, quantified by ESTD, using Aroclor 1254 as the reference standard.

9.3 Total DDT is reported as the sum of p,p'-DDE, p,p'-DDD and p,p'-DDT.

9.4 Annex C, tables 2 and 3 give the RRT_{DDE} for PCB congeners and other OCs.

10. **PRECISION AND ACCURACY**

10.1 The detection limits are: 5 ng/mL (5 pg injected) total PCB and 1 ng/mL (1 pg injected) for PCB congeners and other OC's.

10.2 The precision and accuracy data are given in Annex D.
11. **GLC/MSD CONFIRMATION**

11.1 The confirmation of PCBs and OCs can be done by gas liquid chromatography equipped with a mass spectrometer detector (GLC/MSD). Typical operating conditions are summarized in Table 1:

<table>
<thead>
<tr>
<th>Column</th>
<th>HP MS-5, 30 m, 0.25 mm i.d., 0.25 µm, cross-linked borosilicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier gas</td>
<td>He</td>
</tr>
<tr>
<td>Injector temperature</td>
<td>200 °C/0 min, 90 °C-min., 250 °C Hold</td>
</tr>
<tr>
<td>Pressure program</td>
<td>60 psi/1 min, 60 psi-min, 7.5 psi/0 min, 0.35 psi-min, 17.5 psi Hold</td>
</tr>
<tr>
<td>Column program</td>
<td>120 °C/1 min, 15 °C-min, 165 °C/1 min, 1 °C-min, 185 °C/1 min, 2 °C-min, 250 °C Hold</td>
</tr>
<tr>
<td>Injection volume</td>
<td>1 µL</td>
</tr>
<tr>
<td>Injection technique</td>
<td>viscosity delay 2 sec. for filling the syringe then fast injection</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>280 °C</td>
</tr>
<tr>
<td>SIM</td>
<td>Choose one m/z for quantification and two m/z as qualifiers</td>
</tr>
<tr>
<td></td>
<td>Dwell time: sufficient to capture at least 9 data points</td>
</tr>
</tbody>
</table>

**Table 1: MSD operating conditions**

12. **REFERENCES**


## ANNEX A

### Table 1

<table>
<thead>
<tr>
<th>Pesticides Mixture</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldrin</td>
<td>2,4'-dichlorobiphenyl</td>
</tr>
<tr>
<td>α-BHC</td>
<td>2,2',5-trichlorobiphenyl</td>
</tr>
<tr>
<td>α-BHC</td>
<td>2,4,4'-trichlorobiphenyl</td>
</tr>
<tr>
<td>γ-BHC</td>
<td>2,2',3,5'-tetrachlorobiphenyl</td>
</tr>
<tr>
<td>γ-BHC (lindane)</td>
<td>2,2',5,5'-tetrachlorobiphenyl</td>
</tr>
<tr>
<td>α-chlordane</td>
<td>2,3',4,4'-tetrachlorobiphenyl</td>
</tr>
<tr>
<td>γ-chlordane</td>
<td>3,3',4,4'-tetrachlorobiphenyl</td>
</tr>
<tr>
<td>4,4'-DDD</td>
<td>2,2',4,5,5'-pentachlorobiphenyl</td>
</tr>
<tr>
<td>4,4'-DDE</td>
<td>2,3,3',4,4'-pentachlorobiphenyl</td>
</tr>
<tr>
<td>dieldrin</td>
<td>3,3',4,4',5-pentachlorobiphenyl</td>
</tr>
<tr>
<td>endosulfan I</td>
<td>2,2',3,3',4,4'-hexachlorobiphenyl</td>
</tr>
<tr>
<td>endosulfan II</td>
<td>2,2',3,4,4',5'-hexachlorobiphenyl</td>
</tr>
<tr>
<td>endosulfan sulfate</td>
<td>2,2',4,4',5,5'-hexachlorobiphenyl</td>
</tr>
<tr>
<td>endrin</td>
<td>2,2',3,3',4,4',5-heptachlorobiphenyl</td>
</tr>
<tr>
<td>endrin aldehyde</td>
<td>2,2',3,3',4,4',5,5'-heptachlorobiphenyl</td>
</tr>
<tr>
<td>endrin ketone</td>
<td>2,2',3,4,4',5,6-heptachlorobiphenyl</td>
</tr>
<tr>
<td>heptachlor</td>
<td>2,2',3,3',4,4',5,5,6-heptachlorobiphenyl</td>
</tr>
<tr>
<td>heptachlor epoxide</td>
<td>2,2',3,3',4,4',5,5,6-nonachlorobiphenyl</td>
</tr>
<tr>
<td>methoxychlor</td>
<td>decachlorobiphenyl</td>
</tr>
<tr>
<td>decachlorobiphenyl</td>
<td>decachlorobiphenyl</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>PCB Congeners Mixture</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aroclor 1254</td>
<td>2,4',5-heptachlorobiphenyl</td>
</tr>
<tr>
<td>HCB</td>
<td>2,2',4,5,6-heptachlorobiphenyl</td>
</tr>
<tr>
<td>4,4'-DDDE</td>
<td>2,2',3,4,4'-hexachlorobiphenyl</td>
</tr>
<tr>
<td>mirex</td>
<td>2,2',3,3',4,4',5-heptachlorobiphenyl</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>PCB Standard</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aroclor 1254</td>
<td>2,4',5-heptachlorobiphenyl</td>
</tr>
<tr>
<td>HCB</td>
<td>2,2',4,5,6-heptachlorobiphenyl</td>
</tr>
<tr>
<td>4,4'-DDDE</td>
<td>2,2',3,4,4'-hexachlorobiphenyl</td>
</tr>
<tr>
<td>mirex</td>
<td>2,2',3,3',4,4',5-heptachlorobiphenyl</td>
</tr>
</tbody>
</table>

ANNEX B

Figure 1: CHROMATOGRAPHY COLUMN
Figure 2: FILTER COLUMN

- 35mm
- 130mm
- 50mm
- 20mm DIAMETER, FRITTED GLASS DISC COARSE POROSITY
- 24/40 GROUND-GLASS JOINT
- 8mm
ANNEX C

C.1 Retention times and Relative retention times (RRT\textsubscript{DDE}) to \(p,p'\)-DDE, eluted on a Hewlett Packard Ultra-1 column. 100 % methyl silicone on a cross-linked borosilicate glass-capillary column or similar.

C.1.1 Total PCB's:

<table>
<thead>
<tr>
<th>PEAK NAME</th>
<th>RT</th>
<th>RRT\textsubscript{DDE}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB PEAK 1</td>
<td>24.043</td>
<td>0.642</td>
</tr>
<tr>
<td>PCB PEAK 2</td>
<td>31.131</td>
<td>0.831</td>
</tr>
<tr>
<td>PCB PEAK 3</td>
<td>33.948</td>
<td>0.906</td>
</tr>
<tr>
<td>PCB PEAK 4</td>
<td>36.97</td>
<td>0.987</td>
</tr>
<tr>
<td>DDE (retention time reference)</td>
<td>37.473</td>
<td>1.000</td>
</tr>
<tr>
<td>PCB PEAK 5</td>
<td>40.316</td>
<td>1.076</td>
</tr>
<tr>
<td>PCB PEAK 6</td>
<td>42.655</td>
<td>1.138</td>
</tr>
<tr>
<td>PCB PEAK 7</td>
<td>43.003</td>
<td>1.148</td>
</tr>
<tr>
<td>PCB PEAK 8</td>
<td>45.362</td>
<td>1.211</td>
</tr>
</tbody>
</table>

C.1.2 PCB congeners:

<table>
<thead>
<tr>
<th>PEAK NAME</th>
<th>RT</th>
<th>RRT\textsubscript{DDE}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCB</td>
<td>14.181</td>
<td>0.385</td>
</tr>
<tr>
<td>BZ-28</td>
<td>20.731</td>
<td>0.563</td>
</tr>
<tr>
<td>HEPTACHLOR</td>
<td>22.232</td>
<td>0.604</td>
</tr>
<tr>
<td>ALDRIN</td>
<td>23.901</td>
<td>0.649</td>
</tr>
<tr>
<td>BZ-52</td>
<td>25.975</td>
<td>0.705</td>
</tr>
<tr>
<td>BZ-101</td>
<td>33.797</td>
<td>0.918</td>
</tr>
<tr>
<td>DDE</td>
<td>36.824</td>
<td>1.000</td>
</tr>
<tr>
<td>BZ-118</td>
<td>40.189</td>
<td>1.091</td>
</tr>
<tr>
<td>BZ-153</td>
<td>42.854</td>
<td>1.164</td>
</tr>
<tr>
<td>BZ-138</td>
<td>45.201</td>
<td>1.227</td>
</tr>
<tr>
<td>BZ-180</td>
<td>52.076</td>
<td>1.414</td>
</tr>
<tr>
<td>MIREX</td>
<td>54.224</td>
<td>1.473</td>
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</table>
C.1.3 Organochloride pesticides:

<table>
<thead>
<tr>
<th>PEAK NAME</th>
<th>RT</th>
<th>RRT&lt;sub&gt;DDE&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-BHC</td>
<td>12.993</td>
<td>0.353</td>
</tr>
<tr>
<td>β-BHC</td>
<td>13.785</td>
<td>0.374</td>
</tr>
<tr>
<td>γ-BHC</td>
<td>15.011</td>
<td>0.408</td>
</tr>
<tr>
<td>δ-BHC</td>
<td>15.344</td>
<td>0.417</td>
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<tr>
<td>HEPTACHLOR</td>
<td>25.227</td>
<td>0.685</td>
</tr>
<tr>
<td>ALDRIN</td>
<td>25.967</td>
<td>0.705</td>
</tr>
<tr>
<td>HEPTACHLOR EPOXIDE</td>
<td>29.863</td>
<td>0.811</td>
</tr>
<tr>
<td>γ-CHLORDANE</td>
<td>32.319</td>
<td>0.878</td>
</tr>
<tr>
<td>ENDOSULFANE 1</td>
<td>33.77</td>
<td>0.917</td>
</tr>
<tr>
<td>α-CHLORDANE</td>
<td>34.199</td>
<td>0.929</td>
</tr>
<tr>
<td>DIELDRIN</td>
<td>36.493</td>
<td>0.991</td>
</tr>
<tr>
<td>DDE</td>
<td>36.816</td>
<td>1.000</td>
</tr>
<tr>
<td>ENDRIN</td>
<td>38.33</td>
<td>1.041</td>
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<tr>
<td>ENDOSULFAN 2</td>
<td>38.62</td>
<td>1.049</td>
</tr>
<tr>
<td>ENDRIN ALDEHYDE</td>
<td>40.233</td>
<td>1.093</td>
</tr>
<tr>
<td>DDD</td>
<td>40.528</td>
<td>1.101</td>
</tr>
<tr>
<td>ENDOSULFAN SULFATE</td>
<td>42.859</td>
<td>1.164</td>
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<tr>
<td>DDT</td>
<td>44.73</td>
<td>1.215</td>
</tr>
<tr>
<td>ENDRIN EPOXIDE</td>
<td>46.812</td>
<td>1.272</td>
</tr>
<tr>
<td>METHOXYCHLOR</td>
<td>50.294</td>
<td>1.366</td>
</tr>
<tr>
<td>DECACHLOROBIPHENYL</td>
<td>68.928</td>
<td>1.872</td>
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ANNEX D
QA/QC DATA

Results of homogenized sample of National Check Program (CAPCO), Interdepartmental Check Program (FICP) or other internal quality controls gave the following:

D.1

<table>
<thead>
<tr>
<th>Analyte</th>
<th>PCB</th>
<th>HCB</th>
<th>DDE</th>
<th>Mirex</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppb</td>
<td>848</td>
<td>17</td>
<td>192</td>
<td>49</td>
</tr>
<tr>
<td>R.S.D. in %</td>
<td>7.9</td>
<td>10.9</td>
<td>13.9</td>
<td>11.2</td>
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</tbody>
</table>

Table 1 - Reproducibility

D.2

<table>
<thead>
<tr>
<th>Analyte</th>
<th>PCB</th>
<th>HCB</th>
<th>DDE</th>
<th>Mirex</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppb</td>
<td>1453</td>
<td>19</td>
<td>57</td>
<td>138</td>
</tr>
<tr>
<td>R.S.D. in %</td>
<td>4.2</td>
<td>19</td>
<td>3.7</td>
<td>2.3</td>
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Table 2 - PCB Congeners

D.3

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<tr>
<th>IUPAC #</th>
<th>28</th>
<th>52</th>
<th>101</th>
<th>118</th>
<th>138</th>
<th>153</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppb</td>
<td>19</td>
<td>62</td>
<td>88</td>
<td>114</td>
<td>122</td>
<td>48</td>
<td>14</td>
</tr>
<tr>
<td>% Variation</td>
<td>26</td>
<td>2.6</td>
<td>1.8</td>
<td>1.4</td>
<td>6.6</td>
<td>2.0</td>
<td>15</td>
</tr>
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</table>

Table 3 - PCB Congeners

D.4

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<tr>
<th>IUPAC #</th>
<th>28</th>
<th>52</th>
<th>101</th>
<th>118</th>
<th>138</th>
<th>153</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppb</td>
<td>19</td>
<td>37</td>
<td>70</td>
<td>60</td>
<td>81</td>
<td>85</td>
<td>81</td>
</tr>
<tr>
<td>% Variation</td>
<td>26</td>
<td>10.2</td>
<td>12.4</td>
<td>13.2</td>
<td>13.0</td>
<td>10.3</td>
<td>6.9</td>
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</table>

Table 4 - PCB Congeners
D.5

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<thead>
<tr>
<th>Year</th>
<th># Labs</th>
<th>CAPCO, ng/mL</th>
<th>STD DEV.</th>
<th>This method, ng/mL</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>22</td>
<td>2397</td>
<td>673</td>
<td>2060</td>
<td>16.4</td>
</tr>
<tr>
<td>1991</td>
<td>16</td>
<td>2166</td>
<td>558</td>
<td>2180</td>
<td>0.6</td>
</tr>
<tr>
<td>1992</td>
<td>12</td>
<td>1136</td>
<td>150</td>
<td>1440</td>
<td>21.1</td>
</tr>
<tr>
<td>1993</td>
<td>17</td>
<td>1083</td>
<td>264</td>
<td>1295</td>
<td>16.3</td>
</tr>
<tr>
<td>1994</td>
<td>12</td>
<td>1525</td>
<td>457</td>
<td>1895</td>
<td>19.5</td>
</tr>
<tr>
<td>Average for 5 years</td>
<td>16</td>
<td>1661</td>
<td>420</td>
<td>1774</td>
<td>6.9</td>
</tr>
</tbody>
</table>

Table 5 - Total PCB; data from CAPCO Collaborative Program
CHAPTER 1 - CONTAMINANTS
SECTION 4: SELENIUM

1. SCOPE AND APPLICATION

1.1 This method is applicable to fish tissues and various fish products. Levels in the range between 0.05 and 800 ng may be determined. Higher selenium levels can be measured by making the appropriate sample dilution.

2. PRINCIPLE OF THE METHOD

2.1 The sample is digested using nitric, perchloric and sulfuric acids. Interfering elements are masked with disodium EDTA. The selenium is complexed with 2,3-diaminonaphthalene, extracted into cyclohexane, and determined fluorometrically.

3. INTERFERENCES

3.1 Elements such as iron, copper and vanadium interfere. These are removed by using disodium EDTA.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Commercial shipment: Take a representative sample from the product lot and store as to maintain sample integrity.

4.2 Survey samples: Fish may be either pooled or individual. For species normally greater than 30 cm in length, an individual fish may be used as a sample. For species less than 30 cm in length, a pooled sample is required. Store as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Commercial shipment: Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For fish and fish products that contains no free liquid, comminute the sample until homogeneous.
5.1.2 For products that are packed in water, brine or similar medium that is normally discarded by the consumer; open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

5.1.3 For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil; transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.2 Survey samples:

5.2.1 For individual fish, weigh and measure the fork-length, i.e. from the nose to the fork of the tail, for size correlation.

5.2.2 For a pooled sample, determine the average values for length and weight of the fish.

5.2.3 Pass the skinned fillets through a commercial meat grinder a sufficient number of times to obtain a homogeneous blend (e.g. three times).

5.3 Collect the homogenized sample into a thoroughly cleaned, sealable plastic pot or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. **APPARATUS**

6.1 Fluorometer. Filter fluorometer or spectrofluorometer capable of excitation at 366 nm and detection of fluorescence at 525 nm.

6.2 Cuvets or Pyrex culture tubes, 12 x 75 mm, matched.

6.3 Wrist-action shaker.

7. **REAGENTS**

7.1 Nitric acid (HNO₃).

7.2 Sulphuric acid (H₂SO₄).
7.2.1 Sulphuric acid solution (5N). Dilute 140 mL H₂SO₄ to 1 L with distilled water.

7.3 Perchloric Acid (HClO₄) 70%

7.4 Ammonium hydroxide (NH₄OH).

7.4.1 Ammonium hydroxide solution (ca 6N). Dilute 400 mL NH₄OH to 1 L with distilled water.

7.5 Cyclohexane

7.6 Disodium ethylenedinitrilotetraacetate (EDTA).

7.6.1 EDTA solution (0.02 M). Dissolve 7.445 g Na₂H₂EDTA.2H₂O in distilled water and dilute to 1 L.

7.7 2,3-Diaminonaphthalene (DAN).

7.7.1 2,3-Diaminonaphthalene solution (1 mg/mL). Pulverize DAN in a mortar to pass through an 80-mesh sieve. Insert a glass wool plug in the stem of a 250 mL separatory funnel and add 100 mL 5N H₂SO₄. Transfer 0.10 g DAN to separatory funnel and dissolve in the acid. Add 30 mL cyclohexane and shake 5 minutes. Let phases separate 5 minutes, drain lower phase into another separator and discard cyclohexane (upper) phase. Repeat cyclohexane extraction twice more and, after third extraction, drain lower phase into a low-actinic glass stoppered bottle, add 1 cm of hexane and store in the cold.

7.8 Selenious acid (H₂SeO₃).

7.8.1 Selenium standard solution (0.1 mg/mL). Dissolve 0.1634 g H₂SeO₃ in distilled water and dilute with 0.1N H₂SO₄ to 1 L. Make further dilutions in 0.1N H₂SO₄ as required.

8. PROCEDURE

8.1 Place an accurately weighed sample containing 1.0 g dry matter and 800 ng Se with 3 glass beads into a suitable container (100 mL Kjeldahl flask or 125 mL erlenmeyer flask). Prepare appropriate working standards (such as 0, 0.2, 0.4, 0.6, and 0.8 ug Se) and carry through entire procedure with sample and reagent blank.
8.2 Add 6.0 mL conc HNO₃ and heat cautiously until organic matter is in solution. Take care to prevent severe foaming or bumping. Cool.

8.3 Add 2.0 mL 70% HClO₄ and 5.0 mL conc H₂SO₄ and return to hot plate. Heat until solution turns greenish-yellow, then colorless. (If charring occurs, repeat analysis with new sample, using higher HNO₃/HClO₄/sample weight ratio. If this fails, add small amounts HNO₃ at first signs of darkening.)

8.4 Remove flask from heat, swirl contents carefully up the neck, replace flask on heater and continue heating until solution becomes colorless and white fumes appear.

8.5 Remove flask from heat, let cool, add 1.0 mL 30% H₂O₂, rinsing walls of flask, and swirl until fuming ceases. Resume heating until contents boil briskly and white fumes appear. Repeat addition of H₂O₂ and heating twice more and continue final heating 10 minutes after appearance of white fumes. Let cool.

8.6 Add 25 mL H₂O, rinsing walls and mixing thoroughly. Transfer quantitatively to 250 mL stoppered erlenmeyer flask, using 3 x 10 mL H₂O rinses.

8.7 Add successively with mixing, 10.0 mL EDTA solution, 25.0 mL 6N NH₄OH, and 5.0 mL DAN solution.

8.8 Quickly bring contents to a brisk boil with a bunsen burner, transfer to a hot plat and continue boiling for two minutes.

8.9 Let reaction mixture stand at room temperature for a definite interval between 1½ and 2 hours. Use same interval for sample, standards, and blank.

8.10 Accurately add a suitable quantity of cyclohexane (6 - 10 mL), stopper flask, and place on shaker for 5 minutes.

8.11 Allow layers to separate. Draw upper cyclohexane layer into fluorometer cuvet using a Pasteur pipet. If necessary, centrifuge the solution to remove suspended water droplets.

8.12 Zero fluorometer against reagent blank and read fluorescence of sample and standards.
9. **CALCULATIONS**

9.1 Prepare a calibration curve of fluorescence versus nanograms Se in standards.

9.2 Determine Se content by comparing sample reading with calibration curve. Take into account the sample weight and the dilution factor. Express the result in terms of total selenium on a wet weight basis (ppm).

10. **PRECISION AND ACCURACY**

10.1 NBS bovine liver (SRM 1577) should be analyzed with each set of samples. For twenty-one trials, a value of $1.1 \pm 0.1$ ppm was obtained. The NBS certified value for this material is $1.1 \pm 0.1$ ppm.

10.2 The mean apparent recovery of selenium added as selenite or selenate at 100 and 500 ng levels to 0.1 g fish was 101.0%; the actual recovery of the same levels of selenium from standard solutions was 96.6%. (See reference 12.2).

10.3 The precision and accuracy was deemed acceptable after collaborative study, and the method has subsequently been adopted as official final action by the A.O.A.C. (see references 12.4 and 12.5).

11. **REMARKS**


12. **REFERENCES**


CHAPTER 1 - CONTAMINANTS
SECTION 5: DETERMINATION OF LEAD IN FISH BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

1. SCOPE AND APPLICATION

1.1 This procedure is applicable to the determination of lead in fish at levels of 0 - 10 ug/mL.

2. PRINCIPLE OF THE METHOD

2.1 The samples are dry-ashed at 500°C then dissolved in 1 N HCl. Lead content is determined by atomic absorption spectrophotometry at 283.3 nm.

3. INTERFERENCES

3.1 Large excesses of other elements may interfere with the lead signal (e.g., 10,000 mg/L Fe enhances the lead signal).

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Comminute the sample until homogeneous and place the homogenate in an acid-washed sealable plastic or glass container. Store in a freezer until required. Ensure the sample is homogeneous prior to weighing. If liquid separates from the sample, reblend before use.

6. APPARATUS

6.1 Atomic absorption spectrophotometer double beam, equipped with a deuterium background corrector and capable of absorbance measurements at 283.3 nm and 217 nm, and having a range of 0 - 10 ug/mL.

6.2 Hollow cathode or electrodeless discharge lead lamp.

6.3 Crucible - Porcelain, ca 50 mL capacity and 5 cm deep; or tall form Vycor or quartz beaker, 100 mL (Kontes Glass Co., K319000).

6.4 Muffle furnace, capable of ashing samples at 500°C.
7. REAGENTS

7.1 Hydrochloric acid - 1 N. Dilute 82 ml HCl to 1 L with H$_2$O.

7.2 Nitric Acid - Add 128 mL redistilled HNO$_3$ to 500 - 800 mL distilled or deionized H$_2$O and dilute to 2 L. Redistilled HNO$_3$ (G. Frederick Smith Chemical Co., No. 53) may be diluted and used without redistillation.

7.3 Lead Standard Solutions: (1) Stock Solution - 1000 ug/mL. Dissolve 1.5985g recrystallized Pb(NO$_3$)$_2$ in ca 500 mL 1 N HCl in 1 L volumetric flask and dilute to volume with 1 N HNO$_3$; (2) Working Solution - 10 ug Pb/mL. Pipet 10 mL stock solution into 1 L volumetric flask, add 82 mL HCl, and dilute to volume with H$_2$O.

7.4 Buffer Solution - Disperse 163 g EDTA in 200 mL H$_2$O in 2 L volumetric flask and add enough NH$_4$OH to dissolve. Dilute 60 mL 70.5% HClO$_4$ (CAUTION: SEE REMARKS) by pouring carefully into ca 500 mL H$_2$O and cool. Dissolve 50 g La$_2$O$_3$ in HClO$_4$ solution. Add 8 drops Me orange indicator to ammoniacal EDTA solution and add La$_2$O$_3$ to EDTA solution while stirring vigorously. If necessary, add NH$_4$OH to maintain alky of above solution to methylorange. Dilute to 2 L.

8. PROCEDURE

8.1 Test purity of reagents as follows: Evaporate 4 mL HNO$_3$ (CAUTION: SEE REMARKS) in crucible to dryness on hot plate or steam bath, dissolve residue in 1 N HCl and transfer to 25 mL volumetric flask. Heat residue again successively with two 5 mL portions 1 N HCl and add to flask. Cool, dilute to volume with 1 N HCl and mix. Proceed with determination. Total reagent blank should be < 10 ug Pb (equivalent to 0.4 ppm in sample) for determinations at levels ≥ 1 ppm.

For determinations at < 1 ppm follow the procedure as outlined in Appendix A to attain blank at < 50% of limiting level of concern.

8.2 Weigh ca 25 g (to nearest 0.1 g) sample into crucible. Dry 2 hrs at 135E - 150EC. Transfer to a cold temperature - controlled furnace and slowly raise temperature to 500EC. Set control and maintain temperature at 500EC as temperatures of 550EC and higher may cause loss of Pb. Ash overnight (16 hrs).

8.3 Remove sample, let cool to room temperature, cautiously add 2 mL HNO$_3$ and swirl.
8.4 Evaporate carefully "just" to dryness on "warm" hot plate or steam bath. Transfer to cooled furnace, slowly raise temperature to 500°C, hold at 500°C for 1 hr.

8.5 Remove dish and cool.

8.6 Repeat HNO₃ ashing, if necessary, to obtain clean, practically C-free ash.

8.7 Add 10 mL 1 N HCl and dissolve ash by heating cautiously on hot plate. Transfer to 25 mL volumetric flask.

8.8 Heat ash residue again successively with two 5 mL portions of 1 N HCl and add to flask.

8.9 Cool, dilute to volume with 1 N HCl and mix.

9. Transfer 0, 1, 3, 5, 15, 25 and 50 mL Pb working solution to separate 50 mL volumetric flasks and dilute to volume with 1 N HCl (0, 0.2, 0.6, 1.0, 3.0, 5.0 and 10.0 ug Pb/mL respectively).

9.1 Set spectrophotometer to previously established optimum conditions for maximum signal at 283.3 nm. Use air - C₂H₂ flow rates recommended by manufacturer for standard conditions for Pb.

9.2 a) For digital concentration readout, calibrate in concentration mode with solutions containing 0.2 and 10.0 ug Pb/mL. Record concentration directly after calibration of instrument.

   b) For strip-chart readout, set amplification to give > 1% absorption reading for 0.2 ug/mL working solution and prepare standard curve of absorption against ug Pb/mL.

9.3 Use aliquot of prepared sample 8.2 - 8.9 and proceed as outlined in a) or b).

   a) Clear solutions - Determine absorption of sample and standard solution as outlined in 9.2 a) or b) using the following sequence 3 times:

   Read the standard solution first; then, sample solution, alternating until all sample and standard solutions have been read. When many samples are to be analyzed, standard solutions may be read after a series of 3 samples instead of after each.

   ppm Pb = [(ug Pb/mL sample solution) x 25]/g sample
b) Cloudy solutions - Proceed as for clear solutions, but add 1 mL buffer solution, 7.4, to aliquots of sample and standard solutions before reading.

If additional dilutions are necessary or buffer is added:

\[ \text{ppm Pb} = (\text{ug Pb/mL diluted sample}) \times (\text{mL diluted sample/mL original aliquot}) \times (25/g \text{ sample}) \]

10. **PRECISION AND ACCURACY**

10.1 The precision and accuracy was deemed acceptable after collaborative study, and the method has been adopted as official final action by the AOAC. (See references 12.1 and 12.2).

10.2 The average lead recovery from 6 paired samples at 1-2, 5-6 and 10-11 ppm levels was 100.7%. (See reference 12.3)

11. **REMARKS**

11.1 Wash all glassware with water, soak in nitric acid (1 + 2) followed by distilled water rinses.

11.2 Perchloric Acid - HClO₄

Contact with oxidizable or combustible materials or with dehydrating or reducing agents may result in fire or explosion. Persons using this acid should be thoroughly familiar with its hazards. Safety practices should include the following:

a) Remove spilled HClO₄ by immediate and thorough washing with large amounts of water.

b) Hoods, ducts, and other devices for removing HClO₄ vapor should be made of chemically inert materials and so designed that they can be thoroughly washed with water. Exhaust systems should discharge in safe location and fan should be accessible for cleaning.

c) Avoid use of organic chemicals in hoods or other fume removal devices used for HClO₄ digestions.

d) Use goggles, barrier shields, and other devices as necessary for personal protection; use polyvinyl chloride, not rubber, gloves.

e) In wet combustions with HClO₄, treat sample first with HNO₃ to destroy easily oxidizable organic matter unless otherwise specified. Do not evaporate to dryness.
**f)** Contact of HClO$_4$ with strong dehydrating agents such as P$_2$O$_5$ or concentrated H$_2$SO$_4$ may result in formation of anhydrous HClO$_4$ which reacts explosively with organic matter and with reducing agents. Exercise special care in performing analyses requiring use of HClO$_4$ with such agents. Extremely sensitive to shock and heat when concentration is > 72%.


### 11.3 Nitric Acid

Reacts vigorously or explosively with aniline, H$_2$S, flammable solvents, hydrazine, and metal powders (especially Zn, Al, and Mg). Gaseous nitrogen oxides from HNO$_3$ can cause severe lung damage. Copious fumes are evolved when concentrated HNO$_3$ and concentrated HCl are mixed. Avoid premixing. Use effective fume removal device when fumes are generated. Handle with disposable polyvinyl chloride, not rubber, gloves.

### 12. REFERENCES


12.3 Gajan, Raymond J. and Damon Larry, "Determination of Lead in Fish by Atomic Absorption Spectrophotometry and by Polarography. II. Collaborative Study", Journ. AOAC 55, 733-736 (1972).
APPENDIX A

The analyst should decide whether nature of determination requires unusual care in purification of reagents, or whether blank determination will suffice. The smaller the amount of Pb to be detected, the greater the care required in reduction of blank.

To test suitability of reagents, place 10 - 15 g solid reagents dissolved in redistilled H$_2$O or 15 - 20 mL concentrated acids previously neutralized with redistilled NH$_4$OH in separator and add enough Pb-free citric acid to prevent precipitation by HN$_4$OH of Fe, Al, alkaline, earth phosphates, or other substances. Make solution ammoniacal and add 2-3 mL 10% KCN solution. Shake solution with ca 5 mL dithizone solution, (5 - 10 mg/L). If lower layer is green, transfer it to another separator and extract excess dithizone with NH$_4$OH (1 + 99) to which has been added drop of KCN solution. If CHCl$_3$ layer is colorless, consider test negative for use with dithizone methods.

When special purification becomes necessary, redistil H$_2$O (distilled H$_2$O stored in Sn-lined tanks usually contains Pb and Sn), HNO$_3$, HCl, HBr, Br, and CHCl$_3$ in all-glass (pyrex) or quartz stills (preferably quartz). Prepare NH$_4$OH by distilling ordinary reagent into ice-cold redistilled H$_2$O. If stills are new, steam them out with hot HCl or HNO$_3$ vapors to remove "surface" Pb. (Subsequent distillates may not be totally Pb-free).

Pb(NO$_3$)$_2$ may be purified as follows: Dissolve 20 - 50 g in minimum of hot H$_2$O and cool with stirring. Filter crystals with suction on small buchner, redissolve, and recrystallize. Dry crystals at 100 - 110°C to constant weight. Cool in desiccator and store in tightly stoppered bottle. (Product has no H$_2$O of crystallization and is not appreciably hygroscopic).

Purify citric acid, NaOAc or NH$_4$OAc, Al(NO$_3$)$_3$, Ca(NO$_3$)$_2$, and Na$_2$SO$_4$ by precipitating Pb from their aqueous solutions with H$_2$S, using 5 - 10 mg CuSO$_4$ as coprecipitant (citric acid and Al(NO$_3$)$_3$ solutions require adjustment with HN$_4$OH to pH 3.0-3.5, bromophenol blue indicator). Filter (fritted glass filter is most convenient), boil filtrates 20 min. to expel excess H$_2$S, and refilter if necessary to obtain brilliantly clear solutions. Purify other reagents by recrystallization.

Store redistilled acids or purified solutions of reagents in teflon or conventional polyethylene containers carefully cleaned of surface Pb with hot HNO$_3$. Paraffin-lined bottles may be used for alkaline reagents.

Carefully clean new glass, plastic and chemical ware with hot 10% NaOH solution followed by hot HNO$_3$ and use only for Pb determinations.
In preparation of samples for analysis, avoid Pb contamination. If mixing or grinding is necessary, use porcelain mortar if possible. Avoid use of metal food grinders unless previous experiment has shown that no contamination of sample with Pb or Sn results. If product to be analyzed cannot be thoroughly mixed in its own container, or if composite sample of number of containers is desired, empty into large glass jar or porcelain dish and mix thoroughly with wooden spoon or porcelain spatula. If liquid portion of sample cannot be incorporated into ground solid material to obtain homogeneous mixture, analyze separately. If food is packed in tins having soldered seams (sardines and meats), open tins from bottom to avoid contaminating sample with bits of solder. Avoid sifting in preparation of samples to prevent metallic contamination or segregation of Pb.
CHAPTER 1 - CONTAMINANTS
SECTION 6: OXYTETRACYCLINE EXTRACTION AND ANALYSIS PROCEDURE

1. SCOPE AND APPLICATION

1.1 This procedure is applicable to a wide range of fresh and frozen fish, shellfish and fish products that have been treated with oxytetracycline, or produced with contaminated raw material. The method has been used to determine oxytetracycline (OTC) concentrations in fish ranging from 0.01 µg/g to over 12 µg/g.

2. PRINCIPLE OF THE METHOD

2.1 Fish tissues are blended to homogeneity and the OTC is extracted with 1 N HCl or an extraction solution containing 0.67 N HCl and 0.67 M ammonium sulfate. The extracts are deproteinized with acetonitrile, filtered through a glass wool plug and a portion cleaned up with a methylene chloride:petroleum ether partition.

2.2 OTC is analyzed by reversed-phase liquid chromatography with UV detection at 355 nm. The amount of OTC present is calculated by comparison with prepared standards of known concentrations.

3. INTERFERENCES

3.1 Retinol, the precursor to Vitamin A, has been reported to cause some interferences with the analysis of OTC in other procedures. To date there has not been any evidence to support that it interferes with this procedure.

3.2 In processed products, such as smoked salmon, salmon pâté, and breaded shrimp, there may be some interferences due to the spices or other ingredients used in the formulation. Any problems encountered can generally be alleviated by slight adjustments to the gradient of the routine chromatography or by using the confirmatory HPLC system.

3.3 Lobster muscle tissue generally does not pose any problems during analysis. However, hepatopancreas or tissue contaminated with hepatopancreas can make the routine analysis difficult. Some species of fish (e.g., milkfish, tilapia, and Nile perch) and some tissues (e.g., liver) contain compounds which can interfere with the
chromatography of OTC. These problems generally can be rectified by altering the gradient conditions to increase the residence time of the analytes on the column or by using the confirmatory HPLC system.

4. **SAMPLING PROCEDURE AND STORAGE**

4.1 Fresh or frozen product.

4.1.1 Collect a minimum of 5 animals or enough to yield 150 g of meat for each of 5 sample units.

4.1.2 Samples should be frozen prior to shipping.

4.1.3 Pack the samples in a shipping container with adequate protection against mechanical damage and temperature change.

4.1.4 Place a description of the sample in the package, i.e., date of collection, time, location, species, and any other pertinent data.

4.1.5 Send to the laboratory without delay.

4.2 Canned or packaged product.

4.2.1 Take a representative sample (minimum of 5 units) from the product lot and store so as to maintain sample integrity.

5. **SAMPLE PREPARATION**

5.1 Finfish.

5.1.1 Take precautions to prevent contamination of the fish or fish slices by material on the outside of the outer bag or from the processing area.

5.1.2 Partially thaw the individual subsamples and remove the skin and backbone.

5.1.3 Divide the tissue from each subsample into two approximately equal portions.

5.1.4 Prepare a composite sample by placing one portion of each subsample into a Silent Cutter or food processor and comminuting until homogeneous.
5.1.5 Refreeze the second portion of each subsample in its original plastic bag in case individual analyses are later required. Wash the cutting board, knife and immediate work area to minimize contamination between subsamples.

5.1.6 Remove a significant portion of the composite sample purée from the Silent Cutter and further comminute it in a food processor to ensure better homogeneity.

5.1.7 Store a portion of this sample below -20 °C in a sealable container for later analysis.

5.1.8 Ensure the prepared sample is homogeneous prior to weighing the portion for extraction. If liquid separates on thawing, reblend prior to use.

5.2 Crustaceans.

5.2.1 Remove the meat from the shell, blend until homogeneous and treat as in 5.1.3.

6. APPARATUS

6.1 Polytron homogenizer or equivalent.

6.2 Food processor.

6.3 Silent Cutter or equivalent.

6.4 Pipettors.

6.4.1 Variable volume pipettors with 5 and 10 mL capacity.

6.5 13 mm nylon syringe filters (0.2 µ pore size recommended).

6.6 3 mL disposable syringes.

6.7 HPLC pump system able to generate reliable, rapid binary gradients at flow rates up to 1.5 mL/min. and at pressures of at least 3000 psi.

6.8 Autosampling system able to communicate with the pump and data system and provide up to 500 µL injection volumes either in one injection or by repeated smaller injections.

6.9 Column oven capable of maintaining the column temperature at 50 °C.
6.10 HPLC UV monitor capable of achieving the required sensitivity at 355 nm with minimal refractive index changes. A diode array detector is required for spectral confirmations.

6.11 Data-handling system and recording device.

6.12 Columns:

6.12.1 Routine: Vydac, 201TP54, 25 cm x 4.6 mm i.d. used without a guard column.

6.12.2 Routine: Polymer Laboratories PLRP-S, 15 cm x 4.6 mm i.d. used without a guard column.

6.12.3 Confirmatory: Partisil, 5 ODS-3 25 cm x 4.6 mm i.d. used without a guard column.

6.13 UV spectrophotometer: capable of measuring accurately in the 350 to 370 nm range.

7. REAGENTS

7.1 Methanol, ACS grade.

7.2 Acetonitrile, HPLC grade.

7.3 Petroleum ether, ACS grade.

7.4 Methlyene chloride, ACS grade.

7.5 Glass-distilled water.

7.6 n,n-Dimethylformamide (DMF), ACS grade.

7.7 Oxytetracycline dihydrate.

7.7.1 Stock standard solution: Approximately 10.8 mg of oxytetracycline dihydrate is dissolved in methanol (may take several hours to complete) and diluted to 100 mL. The standard is calibrated spectrophotometrically using procedure 8.1 to determine the precise concentration.

7.7.2 Spiking standard: (0.010 mg/mL). Pipet an appropriate amount of the stock solution into a 100 mL volumetric flask and dilute to volume with methanol.
7.7.3 Working standard: (0.0010 mg/mL). Pipet an appropriate amount of stock solution into a 100 mL volumetric flask and dilute to volume with 0.01 N HCl.

7.7.4 Calibration solutions: (approximately 0.020 mg/mL). Pipet 1.0 mL of stock solution and dilute to 5.0 mL with ethanol in a volumetric flask.

7.8 Hydrochloric acid (HCl).

7.8.1 HCl (1N) solution. 86.2 mL of conc. acid diluted to 1 L with distilled water.

7.8.2 HCl (0.01 N) solution. 1.0 mL of 1 N HCl diluted to 100 mL with distilled water.

7.9 Oxalic acid.

7.9.1 Oxalic acid (0.01 M) solution. Dissolve 0.90 g of oxalic acid in approximately 600 mL of distilled water then dilute to 1 L with distilled water.

7.10 Trifluoroacetic acid (TFA). Reagent grade. **Warning**: very corrosive and relatively toxic and volatile.

7.11 Ammonium sulfate (ACS grade).

7.12 Extraction solution.

7.12.1 Weigh 8.8 g of ammonium sulfate and place in a 1 L volumetric flask; add 55 mL concentrated HCl and make up to volume with deionized water.

7.13 Antifoam B silicone emulsion.

7.14 Sodium decane sulfonate (ACS grade).

7.15 Phosphoric acid.

7.16 Phosphoric acid (0.02 M) plus decane sulfonate (0.01 M).

7.16.1 Pipet 1.3 mL of phosphoric acid and weigh 2.44 g of sodium decane sulfonate into a 1 L volumetric flask. Dilute to volume with glass-distilled water and filter before use (see remarks 11.6).

7.17 Sodium phosphate.
7.17.1 Sodium phosphate (monobasic, 2 N). Dissolve 27.6 g of NaH$_2$PO$_4$.$\text{H}_2$O in distilled water and dilute to 100 mL.

7.18 Mobile phases.

7.18.1 Mobile phase (Routine - Vydac column). Solvent A: Glass-distilled water + 0.1 % TFA. Solvent B: Acetonitrile + 0.1 % TFA.

7.18.2 Mobile phase (Routine - PLRP-S column). Solvent A: 0.01 M sodium decane sulfonate and 0.02 M phosphoric acid. Solvent B: Acetonitrile.

7.18.3 Mobile phase (Confirmatory - Partisil column). Solvent A: 0.01 M oxalic acid + 6% n,n-Dimethylformamide (DMF). Solvent B: Acetonitrile + 6% DMF.

8. PROCEDURE

8.1 Calibration of OTC Standard Solutions.

8.1.1 Measure the absorbance of four identically prepared calibration solutions at 370 nm. Average the absorbance readings to obtain the concentration of the calibration solutions by using Beer's Law (A = εcl) where ε = 343 at 370 nm. The concentration of the stock OTC solution is calculated by reference to the calibration solutions.

8.2 Extraction.

8.2.1 Accurately weigh 25 g of remixed tissue purée into a 250 mL Erlenmeyer flask.

8.2.2 Blend with an homogenizer in three volumes (mL/g) of 1 N HCl or 2 volumes of extraction solution (2-3 drops of antifoam B are required if the extraction solution is used).

8.2.3 Pipet 8.0 mL of the 1 N HCl blend to a 125 mL Erlenmeyer flask containing 32.0 mL of acetonitrile (swirl the sample immediately before transfer to ensure that the sample remains uniform). If the extraction solution has been used quantitatively, transfer the entire contents of the flask to a volumetric flask, allow 5-10 minutes for the foam to break up (additional antifoam B may be required if the extract is very foamy) and dilute to volume with extraction solution. Shake the contents of the flask thoroughly and
centrifuge approx. 50 mL of the extract for 5 minutes at 2000 rpm. Pipet 8 mL of the supernatant to a 125 mL flask containing 32.0 mL of acetonitrile.

8.2.4 Thoroughly mix this suspension and allow to stand for at least five minutes. Filter the supernatant through a glass wool plug into a 50 mL Erlenmeyer flask.

8.2.5 Transfer a 20 mL aliquot to a 125 mL separatory funnel using a disposable tip pipettor.

8.2.6 Add 20 mL each of methylene chloride and petroleum ether and mix well with vigorous shaking.

8.2.7 Allow the phases to separate and draw off the lower layer quantitatively into a graduated container. Adjust the volume to 4.00 mL with distilled water and mix thoroughly.

8.2.8 For convenience and ease of storage, transfer samples to screw-capped culture tubes.

8.2.9 For each series of 10 samples, run an internal check sample, repeat samples, spiked samples and a reagent blank.

8.2.10 Draw approximately 1.5 mL of sample into a 3 mL disposable syringe and filter through a 0.2 µ syringe filter directly into an autosampler vial and cap. **Caution:** see remarks in section 11.9.

8.3 HPLC Analysis, Routine.

8.3.1 Vydac Column: Equilibrate the system for at least 20 minutes at a column oven temperature of 30 °C with 100 % solvent A. Adjust the column oven temperature or gradient conditions to obtain a retention time of the standards of between 14 to 15 minutes.

8.3.1.1 HPLC Conditions:

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Solvent A - Glass distilled water + 0.1 % TFA.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent B</td>
<td>Acetonitrile + 0.1 % TFA</td>
</tr>
<tr>
<td>Gradient</td>
<td>100 % Solvent A for 2 minutes</td>
</tr>
<tr>
<td></td>
<td>75 % Solvent B in 20 minutes</td>
</tr>
<tr>
<td></td>
<td>100 % Solvent A in 2 minutes</td>
</tr>
<tr>
<td></td>
<td>Equilibrate for 8 minutes</td>
</tr>
<tr>
<td>Flow</td>
<td>1.0 mL/min.</td>
</tr>
<tr>
<td>Wavelength</td>
<td>355 nm</td>
</tr>
</tbody>
</table>
8.3.2 PLRP-S Column: Equilibrate the system for at least 20 minutes at a column oven temperature of 40 °C with 82 % solvent A. Adjust the column oven or gradient conditions to obtain a retention time of the standards between 20 and 23 minutes.

8.3.2.1 HPLC Conditions:
Mobile phase - Solvent A - 0.02 M Phosphoric acid and 0.01 M sodium decane sulfonate.
Solvent B - Acetonitrile
Gradient - 82 % solvent A for 3 minutes
60 % solvent A in 25 minutes
Hold for 5 minutes
End time 30 minutes
Flow - 1.0 mL/minute
Wavelength - 358 nm

8.3.3 Calibrate the instrument by repeated injections of the working standard.

8.3.4 Inject 200 µl (500 µl PLRP-S column) of each sample, check sample, 'spiked sample' or blank and measure the resultant peak heights. Peaks are identified by comparison of retention times with recently run standards.

8.4 HPLC Analysis, Confirmatory.

8.4.1 Partisil Column: Equilibrate the system for at least 20 minutes at a column oven temperature of 50 °C with 100 % solvent A. Adjust the column oven temperature or the gradient to obtain retention times of the standards of between 12 to 14 minutes.

8.4.2 HPLC Conditions:
Mobile phase - Solvent A - 0.01 M Oxalic acid + 6 % DMF.
Solvent B - Acetonitrile + 6 % DMF.
Gradient - 100 % Solvent A for 2 minutes
35 % Solvent B in 15 minutes
70 % Solvent B in 5 minutes
100 % Solvent A in 2 minutes
Equilibrate for 8 minutes
Flow - 1.0 mL/min.
Wavelength - 355 nm
Run time - 32 min.

8.4.3 Calibrate the instrument by repeated injections of the working standard.

8.4.4 Spectral confirmations diode array: Compare the UV spectrum of the sample peak believed to be OTC with the spectrum of the standard OTC peak. Scan the spectrum from 301 to 500 nm and correct both peaks for background absorption before the comparison is made.

8.4.5 Inject 200 µL of each sample onto the HPLC column. Follow each sample run with a 'spiked' run consisting of 200 µL of the sample plus 5 µL of working standard. Inject a standard after each sample and sample plus 'spike' pair to ensure proper retention time comparisons.

9. **CALCULATIONS**

9.1 For a single point calibration, measure peak heights of the standards and calculate the average response factor (height units/µg OTC).

9.2 Calculate the average peak height of the duplicate injections of the samples.

9.3 Calculate the level of OTC in the sample using the following formula:

\[ \mu g \text{ OTC/g ww} = \frac{PH \times D}{PHS \times W} \]

- PH - average peak height
- PHS - peak height per µg OTC standard injected
- D - dilution factor
- W - weight of the sample extracted

9.4 A multi-point calibration may also be used but should not be required if samples are analyzed within the linear range of the method (see 11.8).

10. **PRECISION AND ACCURACY**

10.1 Replicate analysis of standard solutions indicates good reproducibility over the range of concentrations studied with percent relative standard deviations ranging from 2.78 to 8.15%.
10.2 Replicate analysis of salmon tissue samples at residue levels of 0.04 to 0.5 µg/g gave percent relative standard deviations from 9.0 to 23 %. Variations are greatest as the limit of detection is approached.

10.3 The detection limit is approximately 0.01 µg/g for the Vydac and Partisil columns and 0.05 µg/g for the PLRP-S column.

11. REMARKS

11.1 Representative chromatograms of an OTC standard and a salmon tissue extract run on a Vydac column are shown in Appendix A, Figure 1 while a similar sample run on the Partisil column is shown in Figure 3. A calibration curve of OTC standards is shown in Figure 2. A UV diode array absorption spectra comparing standard OTC and a salmon sample containing OTC is shown in Figure 4. Other reversed-phase columns should provide similar results.

11.2 Recoveries of OTC added to tissue samples from a variety of fish species analyzed on the Vydac column over a one year period are shown in Table 1. Recoveries ranged from 81 to 97 % for a variety of species. Recoveries of samples spiked at the 0.2 µg/g level and analyzed by the PLRP-S column ranged from 65 to 95 %.

11.3 Initial analyses are performed on sample composites. The individual subsamples are analyzed if the result of the sample composite is in excess of 0.1 µg OTC/g. Samples which approach or exceed this present alert limit should be confirmed either chromatographically or by using diode-array detection.

11.4 The OTC standard should be kept at freezer temperatures during storage.

11.5 The spiking and working standards must be stored between 0-4 °C.

11.6 Take care when filtering the 0.02 M phosphoric acid, 0.01 M sodium decane sulfonate solution as the solution tends to foam while filtering.

11.7 Aqueous extracts are stable for at least one week when stored in the refrigerator at 4 °C.
11.8 Single point standardization can be generally recommended. However, depending on the equipment or columns used, standardization at other levels may be required.

11.9 For certain autosamplers and injectors the HCl sample extracts must be 'buffered' before injection onto the HPLC to prevent rapid column deterioration. 1.0 mL of extract is added to 0.5 mL of 2 N Na$_2$HPO$_4$ and mixed. Load at least 1.0 mL into a 3 mL disposable syringe and filter through a 0.2 µ nylon syringe filter into an autosampler vial.

11.10 A significant portion of the sample to sample variation may reflect the lack of sample homogeneity rather than method variation. The presence of small bones which are virtually impossible to remove during normal sample processing may greatly affect the results if they are not evenly distributed throughout the sample.

11.11 The method can also be applied to the analysis of tetracycline and chlortetracycline in a variety of fish and fish products.

12. REFERENCES


**APPENDIX A**

Table 1. Percent recovery of OTC added to various fish tissue samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OTC added µg/g</th>
<th>% Recovery</th>
<th># of analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon</td>
<td>0.4</td>
<td>85 ± 11</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>97 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>Lobster muscle</td>
<td>0.4</td>
<td>85 ± 11</td>
<td>10</td>
</tr>
<tr>
<td>hepato</td>
<td>0.4</td>
<td>87 ± 9</td>
<td>3</td>
</tr>
<tr>
<td>Pacific salmon</td>
<td>0.4</td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>0.4</td>
<td>86 ± 8</td>
<td>3</td>
</tr>
<tr>
<td>Arctic char</td>
<td>0.4</td>
<td>81</td>
<td>1</td>
</tr>
<tr>
<td>Shrimp</td>
<td>0.4</td>
<td>95 ± 14</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 1. HPLC UV (355 nm) trace of A: standard OTC (19 ng) and B: salmon tissue extract (200 µl) run on a Vydac (25 cm x 4.6 mm i.d., 5µ) column. Operating conditions: flow rate 1.0 mL/min., column oven 30 °C, program: glass-distilled water plus 0.1 % trifluoroacetic acid (0-2 min.) to 75 % acetonitrile plus 0.1 % trifluoroacetic acid (22 min.).
Figure 2. Standard curve for OTC determinations at 355 nm on the Vydac column. Operating conditions as in Figure 1.
Figure 3. HPLC UV (355 nm) trace of A: standard OTC (19 ng) and B: salmon tissue extract (200 µL) run on a Partisil 5 ODS (25 cm x 4.6 mm i.d.) column. Operating conditions: flow rate 1.0 mL/min., column oven 50 ºC, program, 0.01 M oxalic acid plus 6 % dimethylformamide (DMF) (0-2 min.) to 35 % acetonitrile plus 6 % dimethylformamide (DMF) (17 min.) to 70 % acetonitrile plus 6 % dimethylformamide (DMF) (22 min.).
Figure 4. UV diode-array absorption spectra from the peaks of standard OTC (A) and a salmon sample containing OTC (B) run on the Partisil column and scanned in the range of 301-501 nm. Operating conditions as in Figure 3.
CHAPTER 1 - CONTAMINANTS
SECTION 7: ROMET 30 AND TRIBRSSSEN EXTRACTION AND ANALYSIS
PROCEDURES

1. SCOPE AND APPLICATION

1.1 These procedures are applicable to a wide range of fresh and frozen fish and shellfish that have been treated with Romet 30 and Tribrissen, or have come into contact with contaminated material. Method B has also been used in the analysis of spiced and breaded products.

2. PRINCIPLE OF THE METHOD

2.1 Romet 30 is a mixture of sulfadimethoxine (SDM) and ormetoprim (OMP) in a 5:1 ratio while Tribrissen is a 5:1 mixture of sulfadiazine (SDZ) and trimethoprim (TMP).

2.2 Method A is applicable to the determination of the sulfa drugs SDZ and SDM as well as the potentiators OMP and TMP. Method B is applicable to the determination of the sulfa drugs SDZ and SDM only.

2.3 Method A:

2.3.1 Fish tissues are blended to homogeneity and SDM, SDZ, OMP and TMP are extracted with acetonitrile:water (1:1). The extract is clarified via centrifugation and a portion defatted with hexane. The analytes are partitioned into chloroform and concentrated.

2.3.2 SDM, SDZ, OMP and TMP are analyzed by reversed-phase liquid chromatography with UV detection at 285 ± 5 nm. The level of the analytes is calculated by comparison with prepared standards of known concentrations.

2.4 Method B:

2.4.1 Fish tissues are blended with octadecylsilyl-derivatized (C18) silica packing material. The blended C18/muscle tissue matrix is used to prepare a column that is washed with hexane. Sulfonamides are then eluted with methylene chloride. Extracts are analyzed by HPLC using UV or photodiode array detection at 273 nm.
3. INTERFERENCES

3.1 Fishery products that contain added ingredients, such as in patés, and in breaded, smoked, spiced, or marinated products, may contain material that interferes with the HPLC analysis of one or more of the components. Changes to the chromatographic system can sometimes alleviate the problem but in general, analysis of these types of product should be avoided if possible.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Fresh or frozen product.

4.1.1 Collect a minimum of 5 animals or enough to yield 150 g of meat for each of 5 sample units.

4.1.2 Samples should be frozen prior to shipping.

4.1.3 Pack the samples in a shipping container with adequate protection against mechanical damage and temperature change.

4.1.4 Place a description of the sample in the package, i.e., date of collection, time, location, species, and any other pertinent data.

4.1.5 Send to the laboratory without delay.

4.2 Canned or packaged product.

4.2.1 Take a representative sample (minimum of 5 units) from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Finfish.

5.1.1 Take precautions to prevent contamination of the fish or fish slices by material on the outside of the outer bag or from the processing area.

5.1.2 Partially thaw the individual subsamples and remove the skin and backbone.

5.1.3 Divide the tissue from each subsample into two
approximately equal portions.

5.1.4 Prepare a composite sample by placing one half of each subsample into a Silent Cutter or food processor and comminuting until homogeneous.

5.1.5 Refreeze the second portion of each subsample in its original plastic bag in case individual analyses are later required. Wash the cutting board, knife and immediate work area to minimize contamination between subsamples.

5.1.6 If a Silent Cutter has been used, remove a significant portion of the composite sample, purée and further comminute it in a food processor to ensure better homogeneity.

5.1.7 Store a portion of this sample below -20 °C in a sealable container for later analysis.

5.1.8 Ensure the prepared sample is homogeneous prior to weighing the portion for extraction. If liquid separates on thawing, reblend prior to use.

5.2 Crustaceans.

5.2.1 Remove the meat from the shell, blend until homogeneous and treat as in 5.1.3.

6. APPARATUS

6.1 Methods A and B.

6.1.1 Food processor.

6.1.2 Silent Cutter or equivalent.

6.1.3 Pipettors.

6.1.3.1 Variable volume pipettors with 1, 5 and 10 mL capacity.

6.1.4 Rotary vacuum evaporator or equivalent.

6.1.5 Bench model centrifuge capable of up to 3000 rpm (Method A) or 12000 rpm (Method B).

6.1.6 Autosampling system able to communicate with the pump
and data system and provide up to 100 µL injection volumes either in one injection or by repeated smaller injections.

6.1.7 Column oven capable of maintaining column temperatures of up to 50 °C.

6.1.8 HPLC UV monitor capable of achieving the required sensitivity between 270-290 nm with minimal refractive index changes.

6.1.9 Data-handling system and recording device.

6.2 Method A.

6.2.1 Polytron homogenizer or equivalent.

6.2.2 N₂ evaporator or equivalent.

6.2.3 13 mm nylon syringe filters (0.2 µ pore size recommended).

6.2.4 3 mL disposable syringes.

6.2.5 HPLC pump system able to generate reliable, rapid binary gradients at flow rates up to 1.5 mL/min and at pressures of at least 3000 psi.

6.2.6 Columns.

6.2.6.1 Routine column: Vydac, 201TP54, 25 cm x 4.6 mm i.d. used without a guard column.

6.2.6.2 Alternate column: Partisil 5, ODS-3, 25 cm x 4.6 mm i.d. used without a guard column.

6.3 Method B.

6.3.1 HPLC pump system able to generate reliable, ternary gradients at flow rates up to 1.5 mL/min and at pressures of at least 3000 psi.

6.3.2 100 mL glass mortar and pestle.

6.3.3 10 mL glass syringe barrels.

6.3.4 Filter paper No. 1, 1.5 cm.
6.3.5 Solid-phase extraction manifold.
6.3.6 Turbovap concentrator or equivalent.
6.3.7 Ultrasonic bath.
6.3.8 Routine column: μBondapak 3.9 x 300 mm used with a μBondapak guard column.

7. REAGENTS
7.1 Methods A and B.
7.1.1 Acetonitrile, HPLC grade.
7.1.2 Glass-distilled or deionized water.
7.1.3 Hexanes, ACS grade.
7.2 Method A.
7.2.1 Chloroform, ACS grade.
7.2.2 SDM, SDZ, OMP and TMP Standards.
7.2.2.1 Stock standards (0.1 mg/mL): Weigh 0.0100 g of each standard into separate 100 mL volumetric flasks. Dissolve the standard and bring each flask to volume with acetonitrile. TMP is slow to dissolve in acetonitrile and a drop or two of trifluoroacetic acid may be required to ensure complete dissolution.
7.2.2.2 Working standards (0.0010 mg/mL): Pipette 1.0 mL of each stock standard solution into separate 100 mL flasks and dilute each to volume with glass-distilled water:acetonitrile (2:1).
7.2.2.3 Mixed standards (0.0010 mg/mL): Pipette 1.0 mL of each of SDZ, SDM, TMP and OMP stock standard solutions into a 100 mL flask and dilute to volume with glass-distilled water:acetonitrile (2:1).
7.2.3 Mobile phase.
7.2.3.1 Solvent A: glass-distilled water. Solvent B: Acetonitrile + 0.1 % TFA.
7.3 Method B:

7.3.1 Acetic acid, reagent grade.

7.3.1.1 Acetic acid (0.01 M). Pipet 0.6 mL acetic acid into a 1 L volumetric flask and dilute to volume with deionized water.

7.3.2 Methylene chloride, ACS grade.

7.3.3 C18 - 40 µm 18 % load, endcapped, octadecylsilyl-derivatized silica (Varian Analytichem Bondesil part # 1221-3012 - other brands tested gave unacceptable results).

7.3.3.1 Prepare C18 by washing in a glass column with 2 volumes each of hexane, methylene chloride, and methanol. Dry the C18 in the glass column using a vacuum source. Store the dried C18 in a sealed container until used.

7.3.4 Sulfamerazine (Internal Standard).

7.3.4.1 Sulfamerazine (1 mg/mL) stock solution. Weigh 100 mg sulfamerazine, dissolve in methanol and dilute to 100 mL in a volumetric flask.

7.3.4.2 Sulfamerazine (50 µg/mL) internal standard solution. Pipet 5 mL of stock sulfamerazine into a 100 mL volumetric flask and dilute to volume with methanol.

7.3.5 SDZ, SDM.

7.3.5.1 Stock standards (1 mg/mL): Weigh 100 mg SDZ and SDM into separate 100 mL flasks and dilute to volume with methanol.

7.3.5.2 Sulfa mixed standard (10, 25 and 50 µg/mL). Into 3 separate 100 mL volumetric flasks, pipet 1, 2.5 and 5 mL stock SDM and 1, 2.5 and 5 mL stock SDZ and dilute to volume with methanol.

7.3.5.3 Sulfa mixed working standards. Into 4 separate 100 mL volumetric flasks pipet 0.2, 0.4, 1.0 and 2.0 mL 50 µg/mL mixed sulfa standard and 2.0 mL 50 µg/mL internal standard and dilute to volume with 0.01 M acetic acid. This represents 0.1, 0.2, 0.5, and 1 µg/mL of each standard and 1 µg/mL of internal standard.
8. PROCEDURE

8.1 Method A.

8.1.1 Accurately weigh 5 g of remixed tissue puree into a 50 mL polyethylene centrifuge tube.

8.1.2 Add 20 mL of acetonitrile:glass-distilled water (1:1) and blend until uniform.

8.1.3 Centrifuge for 10 minutes at 3000 rpm.

8.1.4 Transfer a 10 mL aliquot of the supernatant with a disposable-tipped pipettor into a 50 mL glass centrifuge tube.

8.1.5 Extract with 2.0 mL of hexane, centrifuge for 5 minutes at 1000 rpm and draw off and discard the hexane layer.

8.1.6 Add 20 mL of chloroform, mix well and centrifuge for 5 minutes at 1000 rpm.

8.1.7 Draw off the upper aqueous layer and discard.

8.1.8 Quantitatively transfer the lower layer into a 50 mL round-bottomed flask with a small amount of CHCl₃.

8.1.9 Evaporate to dryness on a rotary evaporator (max. bath temp. 40 °C) or equivalent.

8.1.10 Add approximately 10 mL of methanol and re-evaporate to dryness.

8.1.11 Dissolve in 1.0 mL of glass-distilled water:acetonitrile (2:1) and store in the refrigerator at 0-4 °C in screw-topped culture tubes for HPLC analysis.

8.1.12 For each series of 10 samples run an internal check sample, repeat samples, spiked samples and a reagent blank.

8.1.13 Draw the sample into a 3 mL disposable syringe and filter through a 0.2 µm syringe filter directly into an autosampler vial and cap.
8.1.14  HPLC analysis.

8.1.14.1 Vydac column.

Equilibrate the system for at least 20 minutes at a column oven temperature of 35 °C with 100 % solvent A. Adjust the column oven temperature or gradient conditions to obtain baseline separation of all of the standards.

8.1.14.1.1 HPLC conditions.

Mobile Phase - Solvent A: Glass-distilled water
Solvent B: Acetonitrile + 0.1 % TFA
Gradient - 100 % solvent A for 0.1 minutes
8 % solvent B at 0.1 minutes
15 % solvent B at 7 minutes
70 % solvent B at 24 minutes
100 % solvent A in 2 minutes
Equilibrate for 6 minutes
Flow - 1.0 mL/min.
Wavelength - 285 ± 5 nm
Run time - 32 minutes


Equilibrate the system for at least 20 minutes at a column oven temperature of 35 °C with 25 % solvent B. Adjust the column oven temperature or gradient conditions to obtain baseline separation of all the standards.

8.1.14.2.1 HPLC conditions.

Mobile phase - Solvent A: Glass-distilled water
Solvent B: Acetonitrile + 0.1 % TFA
Gradient - 25 % solvent B for 0 minutes
75 % solvent B in 20 minutes
25 % solvent B in 2 minutes
Equilibrate for 6 minutes
Flow - 1.0 mL/min.
Wavelength - 285 ± 5 nm
Run time - 32 minutes

8.1.14.3 Calibrate the instrument by repeated injections of the working standard.
8.1.14.4 Inject 50 µL of each sample, check sample, 'spiked sample' or blank and measure the resultant peak heights. Peaks are identified by comparison of retention times with recently run standards.

8.1.14.5 Spectral confirmations: Compare the UV spectrum of the sample peak(s) in question with the standard peak. Scan the spectrum from 210 nm to 350 nm and correct peaks for background absorption before the comparison is made.

8.2 Method B.

8.2.1 Weigh 0.50 g of tissue into a 100 mL glass mortar. Add 10 µL of 50 µg/mL sulfamerazine solution (internal standard) and 10 µL methanol. For spiked samples use 10 µL of the appropriate spiking solution instead of the methanol.

8.2.2 Add 2 g of washed C18 to the glass mortar. Blend the tissue and C18 with a glass pestle until homogeneous.

8.2.3 Transfer the blended sample-C18 into a 10 mL syringe barrel containing 2 filter paper disks. Place 2 more filter disks on top of the sample-C18 mixture. Compress the mixture until it is packed solidly (about 3 to 4 mL) using a syringe plunger with rubber end and pointed plastic tip removed.

8.2.4 Place the syringe barrel on a vacuum manifold. Add 8 mL hexane to the syringe barrel; with the vacuum on, start flow at a rate of 1-2 drops per second (3-6 mL/minute). After all the hexane has passed through, stop the flow.

8.2.5 Place test tubes (16 mm x 100 mm) in the rack and place in the manifold to collect. Add 8 mL methylene chloride to the syringe barrel; with vacuum on, start flow at a rate of 1-2 drops per second, collect the eluate. After all the methylene chloride has passed through, stop the flow.

8.2.6 Place the test tube containing the methylene chloride extract in the Turbovap and evaporate under nitrogen at max. 45 °C.

8.2.7 To the dry extract add 50 µL methanol and 200 µL 0.01 M acetic acid and mix using a vortex mixer. Place the test tube containing the sample in an ultrasonic bath for 5
minutes. Transfer the sample to a centrifuge tube and centrifuge for 5 minutes at 12000 rpm. Remove the lower portion for analysis by HPLC (any fat remaining will be in the upper layer). Do not filter.

8.2.8 HPLC conditions:

\[
\begin{align*}
\text{t} = 0 \text{ min.} & \quad 80 \% \ 0.01 \text{ M acetic acid} \\
& \quad 10 \% \text{ acetonitrile} \\
& \quad 10 \% \text{ methanol} \\
\text{Linear gradient to:} & \\
\text{t} = 15 \text{ min.} & \quad 30 \% \ 0.01 \text{ M acetic acid} \\
& \quad 60 \% \text{ acetonitrile} \\
& \quad 10 \% \text{ methanol}
\end{align*}
\]

Run time:
18 min.
Flow: 1 mL/min.
Wavelength: 273 nm
Injection volume: 50 µL (photodiode array detection)

9. CALCULATIONS

9.1 Method A.

9.1.1 Measure the peak height (peak area) of each analyte from the standard injections, and calculate the average response factor (height units/µg) for each analyte.

9.1.2 Calculate the average peak height of the duplicate injections of the samples.

9.1.3 Calculate the level of SDM, SDZ, OMP or TMP in the sample using the following formula:

\[
\mu g/g \text{ ww} = \frac{PH \times D}{PHS \times W}
\]

where:

- \(PH\) = Average peak height of the analyte peak in the sample
- \(PHS\) = Response factor of the standard in height units per µg
- \(W\) = Weight of the sample extracted
- \(D\) = Dilution factor

9.2 Method B.

9.2.1 Prepare a calibration curve by plotting the peak area
9.2.2 Determine the sulfadimethoxine and sulfadiazine concentration in the fish tissue by using the following equation:

\[ C_1 \text{ (µg/g tissue)} = \frac{C_2}{W} \]

where:
- \( C_1 \) = sulfa concentration in tissue sample (µg/g)
- \( C_2 \) = sulfa amount in the sample solution (µg)
- \( W \) = weight of tissue (0.5 g)

10. PRECISION AND ACCURACY

10.1 Method A.

10.1.1 Replicate analysis of salmon tissue at residue levels ranging from 0.03 to 0.19 µg/g gave percent relative standard deviations from 1.5 to 12. Variations were the highest as the limit of detection was approached.

10.1.2 The limit of detection in the tissue is approximately 0.01 µg/g for SDM, 0.02 µg/g for OMP and SDZ and between 0.02 and 0.03 for TMP.

10.2 Method B.

10.2.1 The method gives a detection limit of 0.05 µg/g for sulfadimethoxine and sulfadiazine.

11. REMARKS

11.1 Method A (Tables and Figures - Appendix B).

11.1.1 Representative chromatograms of standard SDM, SDZ, OMP and TMP and spiked and control salmon tissue extracts run on a Vydac column are shown in Figures 1 and 2. A chromatogram of SDM, SDZ, OMP and TMP standards run on a Partisil column is shown in Figure 3, while standard curves for the components of Romet 30 and Tribrissee are
shown in Figures 4 and 5.

11.1.2 Recoveries of SDZ, SDM, OMP and TMP from fortified samples of salmon tissue are shown in Table 1. Mean recoveries from tissue samples fortified at the 0.05 to 2.0 µg/g levels were 90, 70, 73 and 85 % for SDZ, TMP, OMP and SDM respectively.

11.1.3 Initial analyses are performed on sample composites. The individual sub-samples are analyzed if the result of the sample composite is in excess of the regulatory limit for any of the components. Samples which approach or exceed this present alert limit should be confirmed either chromatographically and/or by using diode array detection.

11.1.4 The quality of water for the mobile phase is important. Impurities in the water can cause peaks in the chromatograms that interfere with the quantitation of OMP.

11.1.5 Romet 30 and Tribrissen stock, and working standards must be stored in a refrigerator between 0-4 °C.

11.2 Method B (Figures - Appendix B).

11.2.1 Representative chromatograms of a standard solution (1 µg/mL sulfadiazine, sulfamerazine, and sulfadimethoxine), a salmon tissue extract and a spiked salmon tissue extract run on a µBondapak column are shown in Figures 1, 2, and 3 respectively.

11.2.2 Recoveries for sulfadimethoxine at 0.2 µg/g averaged 87 % with a standard deviation of 11.9 %; for sulfadiazine at 0.2 µg/g averaged 97 % with a standard deviation of 8.7 % (n=35). These spiked tissues were 90 % salmon and the remainder other species (mainly shrimp).

11.2.3 Other sulfa drugs (sulfathiazole, sulfamethazine, sulfamethoxine, and sulfisoxazole) can be determined by this method; however, interference from the fish tissue matrix has not been explored for these compounds.

11.2.4 The relative retention time for sulfadiazine is 0.88 and for sulfadimethoxine is 1.44 when compared to the internal standard sulfamerazine.
11.2.5 It is not possible to do a reagent blank with this method; tissue must be used to produce acceptable results as without tissue the chromatograms are very poor.

11.2.6 Filtering the extract tends to give poor recoveries (cause unknown); this does not appear to affect the life of the column.

12. REFERENCES


Table 1: Percent recovery of SDZ, SDM, OMP and TMP added to salmon muscle tissue using Method A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fortification Level (µg/g)</th>
<th>Percent Recovery</th>
<th>SDZ</th>
<th>TMP</th>
<th>OMP</th>
<th>SDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>90</td>
<td>64</td>
<td>70</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
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<td>75</td>
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</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>89</td>
<td>70</td>
<td>70</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>80</td>
<td>65</td>
<td>61</td>
<td>83</td>
<td></td>
</tr>
</tbody>
</table>

Mean recoveries: 90 70 73 85
Figure 1: Liquid chromatogram of 50 ng of each of SDZ, TMP, OMP and SDM. Chromatographic conditions: column: Vydac 201TP54 (25 cm x 4.6 mm i.d.); mobile phase: glass- distilled water (A) and acetonitrile + 0.1 % trifluoroacetic acid (B). System stabilized at 35 °C with 100 % solvent A at a flow rate of 1.0 mL/min. Held for 0.1 min then to 8 % B. Gradient immediately started to 15 % B at 7 min. then to 70 % B in 24 min. UV detection at 288 nm.
Figure 2: Liquid chromatograms of: (A) control salmon tissue sample spiked at the 1.0 µg/g level; and (B) a control salmon tissue sample. Chromatographic conditions as in Figure 1.
Figure 3: Liquid chromatogram of 50 ng of each of SDZ, TMP, OMP and SDM. Chromatographic conditions: column: Partisil 5 ODS-3 (25 cm x 4.6 mm i.d.); mobile phase: glass-distilled water (A) and acetonitrile + 0.1% trifluoroacetic acid (B). System stabilized with 25% B in A at 35 °C with a flow rate of 1.0 mL/min. Gradient immediately started to 75% B at 20 mins. UV detection at 280 nm.
Figure 4: Standard calibration curve for SDM and OMP (Romet 30)
Figure 5: Standard curve for SDZ and TMP (Tribrissen)
Figure 1: Liquid chromatogram of 50 ng of each of sulfadiazine, sulfamerazine, and sulfadimethoxine. Chromatographic conditions: column: µBondapak (3.9 mm x 300 mm); mobile phase: 0.01 M acetic acid (A), methanol (B), and acetonitrile (C). Initial conditions 85 % A, 10 % B, and 5 % C. Gradient started at t=0 min. continuing to t=15 min.: conditions 30 % A, 10 % B, and 60 % C. End time 18 min. Column temperature 30 °C. UV detection at 273 nm.
Figure 2: Liquid chromatogram of a control salmon tissue containing sulfamerazine internal standard. Chromatographic conditions are as in Figure 1.
Figure 3: Liquid chromatogram of a salmon tissue containing internal standard and spiked with 0.2 µg/g sulfadiazine and 0.2 µg/g sulfadimethoxine. Chromatographic conditions as in Figure 1.
CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 1: ASH

1. SCOPE AND APPLICATION

1.1 This method is applicable to fish, fish products, and other materials with a low carbohydrate content.

2. PRINCIPLE OF THE METHOD

2.1 This method consists of oxidizing all organic matter in a weighed sample of the material by incineration and determining the weight of the ash remaining.

3. INTERFERENCES

3.1 Not applicable.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For fish and fish products that contains no free liquid: comminute the sample until homogeneous.

5.1.2 For products that are packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

5.1.3. For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.
5.1.4 For fish meal: grind the sample in a mill or other suitable apparatus until it will pass through a no. 20 sieve.

5.2 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. APPARATUS

6.1 Crucibles, 50 mL, porcelain or Vycor.
6.2 Muffle furnace.
6.3 Desiccator.

7. REAGENTS

7.1 No reagents required.

8. PROCEDURE

8.1 Accurately weight ca 5 g of sample in a crucible which has been ignited and tared. Place crucible in drying oven at 100°C for 24 hours. Transfer to cool muffle furnace and increase the temperature step wise to 550°C ± 5°C. Maintain temperature for 8 hours or until a white ash is obtained. If white ash is not obtained after 8 hours, moisten ash with distilled water, slowly dry on a hot plate, and re-ash at 550°C to constant weight. Repeat if necessary. Remove crucible to a desiccator and weight soon after cool.

9. CALCULATION

9.1 Calculate the percentage ash content (wet weight basis) as follows:

\[
\% \text{ ASH} = \frac{\text{wt. crucible and ash} - \text{wt. crucible}}{\text{wt. crucible and sample} - \text{wt. crucible}} \times 100
\]

9.2 Calculation of ash content on dry basis (when moisture content is known) as follows:

\[
\% \text{ ASH (dry)} = \frac{\% \text{ ash (wet)}}{100 - \% \text{ moisture}} \times 100
\]
10. PRECISION AND ACCURACY

10.1 From the 1979 and 1981 collaborative studies, the following data was found for the precision of the method. The mean results are on the dry basis.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herring Meal</td>
<td>18.47%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrap Meal</td>
<td>20.16%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Herring Meal</td>
<td>15.86%</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Scrap Meal</td>
<td>22.83%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meal</td>
<td>22.13%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried Seal Meal</td>
<td>4.20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pickled Mackerel Fillet</td>
<td>31.32%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cod Fillet &amp; Pickled Mackerel (1 + 1)</td>
<td>24.31%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Coefficient of variation:
- 1.05%
- 0.2%
- 0.7%
- 1.2%
- 0.6%
- 0.3%
- 1.58%
- 0.8%

The average coefficient of variation is ±0.8%.

11. REMARKS

11.1 Use 2.5 g of sample in the case of products which have a tendency to swell.

11.2 Too high a temperature may cause the volatilization of certain elements (particularly K, Na, Cl, and P) and may also cause the mineral matter to melt and fuse.

12. REFERENCES


CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 2: MOISTURE AND VOLATILE MATTER

1. SCOPE AND APPLICATION

1.1 This method is applicable to fish and fish products, including frozen scallops, both IQF and block frozen, and fish by-products.

2. PRINCIPLE OF THE METHOD

2.1 The moisture and low volatile materials are removed by heating at 95-100 °C under partial vacuum.

3. INTERFERENCES

3.1 There are no known significant interferences.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For products that are packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

5.1.2 For products that are packed in a medium that may be or is normally used by the consumer, e.g., fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.1.3 For smoked fish and smoked fish products: remove the sample from the container with forceps or other suitable instrument, place on a coarse screen and allow to drain for ca 5 minutes. Place the sample on a paper towel or other clean absorbent paper and blot. Repeat blotting using a
second and third piece of towel. Comminute the sample until a homogeneous blend is obtained. If the sample texture is too tough for homogenization, pass the sample through a grinder a sufficient number of times to obtain a uniform mix. A food chopper may suffice as an initial step if the sample size is large.

5.1.4 For brined or salt-cured fish: scrape off as many loose salt crystals as possible with a spatula, if any are present, then wipe off any remaining salt crystals or moisture with a paper towel. Comminute the sample until a homogeneous blend is obtained.

5.1.5 For raw fish, fresh or thawed: pass the sample through a grinder a sufficient number of times to obtain a homogeneous blend.

5.1.6 For processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix.

5.1.7 For frozen scallops: obtain five sample units, remove surface glaze from the scallops under running water until no ice can be felt on the surface of the scallops but it is evident that the ice crystals remain within the product; the interior of the product remains frozen. Block frozen product should be gently broken up to individual scallops or scallop pieces and ice within the block should be removed until the surface of the product is free of ice. Place the scallops on a sieve of appropriate size and drain for 1 to 1k minutes. Obtain a total of approximately 100 g of scallop meat from the five sample units. Comminute the 100 g sample until a homogenous blend is attained.

5.2 Collect the homogenized sample into a clean, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, reblend before use.

6. **APPARATUS**

6.1 Vacuum oven.

7. **REAGENTS**
7.1 No reagents required.

8. PROCEDURE

Accurately weigh a moisture dish of appropriate size. Add approximately 10 g of the comminuted sample and reweigh. Place the container in a vacuum oven at 100 °C and less than 100 mm Hg for approximately 5 hours. Remove dish from the oven, cover, cool in desiccator, and weigh. Redry 1 hr and repeat process until constant weight has been achieved, i.e., change in weight between successive dryings at 1 hour intervals is < 5 mg.

9. CALCULATION

9.1 Moisture = \( \frac{100(P-a)}{P} \) percent

\( P \) = weight in g of sample  
\( a \) = weight in g of dried sample

10. REMARKS

10.1 Since salted samples have a tendency to foam, care should be taken to see that the dish is not over-filled.

11. REFERENCES


CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 3: PROTEIN DETERMINATION BY KJELDHAL

1. SCOPE AND APPLICATION
1.1 This method is applicable to fish, fish products, and fish by-products.

2. PRINCIPLE OF THE METHOD
2.1 In the presence of sulfuric acid, sodium sulphate and a catalyst, the amino nitrogen of many organic materials is converted to ammonium sulphate. The ammonia is distilled from an alkaline medium and absorbed in standardized mineral acid. The ammonia is determined by back titration with a standardized mineral base.

3. INTERFERENCES
3.1 Ammonium compounds, chitin, urea, amino acids, and more complex breakdown products of proteins will also be determined as protein unless removed prior to the analysis.

4. SAMPLING PROCEDURE AND STORAGE
4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION
5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.
5.1.1 For fish and fish products that contain no free liquid: comminute the sample until homogeneous.
5.1.2 For products that are packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.
5.1.3 For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.1.4 For fish meal: grind the sample in a mill or other suitable apparatus until it will pass through a no. 20 sieve.

5.2 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. APPARATUS

6.1 Kjeldahl digestion and distillation assembly.

6.2 Kjeldahl digestion flasks, 800 mL.

7. REAGENTS

7.1 Sulfuric acid (H₂SO₄), nitrogen-free.

7.2 Cupric Sulphate (CuSO₄), nitrogen-free, anhydrous.

7.3 Sodium Sulphate (Na₂SO₄), nitrogen-free, anhydrous.

7.4 Sodium Hydroxide (NaOH).

7.4.1 NaOH solution (50% w/v).

7.4.2 NaOH standard solution (0.1 or 0.2N). Prepare as per procedure 50.034 and standardize against potassium hydrogen phthalate as per procedure 50.035 of the A.O.A.C. Official Methods of Analysis, 12th Edition (1975).

7.5 Boiling granules, selenized. Hengar granules are suitable.

7.6 Hydrochloric acid (HCl).

7.6.1 HCl standard solution (0.1N). Standardize against 0.1 or 0.2N NaOH standard solution.
7.7 Conway indicator.

7.7.1 Stock solution. Mix 200 mL of 0.1% Methyl Red solution (in 50% ethanol) with 50 mL of 0.1% Methylene Blue solution (in 50% ethanol).

7.7.2 Working solution. Dilute 1 volume of stock with 1 volume of absolute ethanol and 2 volumes of distilled water. (pH change 5.4: Acid - Purple, Alkaline - Green).

8. PROCEDURE

8.1 Accurately weigh a suitable quantity of fine-grained material (ca 1.2 g for fishmeal, ca 2.5 g for solubles or homogenized fish) and place in digestion flask.

8.2 Add sequentially 15 g Na₂SO₄, 1 g CuSO₄, one or two selenized boiling granules and 25 mL of conc H₂SO₄ to the flask.

8.3 Digest until solution is almost colourless or light green (2 hrs for inorganic material) and then at least a further 30 minutes.

8.4 Cool (do not allow to solidify), and cautiously add 200 mL water. Add additional boiling granules (if necessary) to prevent bumping.

8.5 Pipette 100 mL 0.1N HCl into a 500 mL erlenmeyer flask, add 1 mL Conway's indicator and place the flask under the condenser ensuring that the condenser tip is immersed in the acid solution.

8.6 Tilt the Kjeldahl flask containing the digested sample and add 100 mL of 50% NaOH solution without agitating. Immediately connect the flask to the distilling bulb of the distillation apparatus. Rotate flask to thoroughly mix contents.

8.7 Heat until all ammonia has passed over into the standard acid. Collect approximately 150 mL. Caution, flask will bump. Remove immediately.

8.8 Wash tip of condenser and titrate excess standard HCl in distillate with NaOH standard solution.
9. **CALCULATIONS**

9.1 Calculate the percentage nitrogen (wet weight basis) as follows:

\[
\text{% NITROGEN (wet)} = \frac{(A - B) \times 1.4007}{\text{weight (g) of sample}}
\]

where

\[
A = \text{vol. (mL) std. HCl x normality of std. HCl}
\]

\[
B = \text{vol. (mL) std. NaOH x normality of std. NaOH}
\]

9.2 Calculation of nitrogen content on dry basis (when moisture content is known) is as follows:

\[
\text{% NITROGEN (dry)} = \frac{\text{% Nitrogen (wet)}}{100 - \text{% Moisture}} \times 100
\]

9.3 Calculate the percentage protein (wet or dry basis) as follows:

\[
\text{% PROTEIN} = \text{% nitrogen} \times 6.25
\]

where 6.25 is the protein-nitrogen conversion factor for fish and fish by-products.

10. **PRECISION AND ACCURACY**

10.1 From the 1979 and 1981 collaborative studies, the following data was found for the precision of the method. The mean results are on the dry basis.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5 Mixed Fish Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>68.4%</td>
<td>65.4%</td>
<td>65.8%</td>
<td>68.2%</td>
<td>67.3%</td>
</tr>
<tr>
<td>Coeff. of variation</td>
<td>±1.4%</td>
<td>±0.8%</td>
<td>±0.6%</td>
<td>±1.8%</td>
<td>±0.6%</td>
</tr>
</tbody>
</table>

| 6 Dried Seal Meat  | 85.5% | ±1.2% |
| 7 Pickled Mackerel Fillet | 30.0% | ±1.6% |
| 8 Cod Fillet & Pickled Mackerel (1 + 1) | 47.7% | ±1.7% |

The average coefficient of variation is ±1.2%.
11. **REMARKS**

11.1 Volume of standardized HCl used in distillation may be varied according to the expected nitrogen content of the sample.

11.2 Pour 50% sodium hydroxide solution slowly down the side of the Kjeldahl flask so that it forms a layer underneath the digestion mixture.

11.3 Do not heat any part of the Kjeldahl flask above the level of the digestion mixture.

11.4 Provide adequate ventilation for the removal of fumes during digestion.

11.5 Certain species of fish such as dogfish contain non-protein nitrogen; therefore, when analysing these species use A.O.A.C. procedure 7.024 (12th Edition) for non-protein nitrogen to correct the results.

11.6 Prolonged boiling and too rapid distillation of acid during digestion should be avoided as loss of ammonia may occur.

12. **REFERENCES**


CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 4: SALT (CHLORINE AS SODIUM CHLORIDE) IN SEAFOOD

1. SCOPE AND APPLICATION

1.1 This method is applicable to the determination of the percent salt (NaCl) in all fish and fish products, including smoked fish and fish products packed in a container that has been sealed to exclude air and, with slight modifications, to brine solutions.

2. PRINCIPLE OF THE METHOD

2.1 For fish and fish products, chloride is precipitated by the addition of a slight excess of standardized silver nitrate. The organic matter is oxidized by heating with nitric acid, and the excess silver nitrate is determined by titration with standardized thiocyanate using a saturated solution of ferric ammonium sulphate as the indicator.

2.2 For brine solutions, the chloride is titrated directly with standardized silver nitrate using potassium chromate as the indicator.

3. INTERFERENCES

3.1 For samples digested with nitric acid and analysed by the back-titration method, the only interferences are bromide, iodide, and cyanide which register as equivalent chloride concentrations.

3.2 For aqueous samples, sulphide, thiosulphate and sulphite ions interfere. Remove by adding 0.5 mL of 30 % hydrogen peroxide and allow to stand for one minute before titrating.

3.3 For aqueous samples, orthophosphate in excess of 25 mg/L interferes by precipitating as silver phosphate.

3.4 For aqueous samples, iron in excess of 10 mg/L interferes by masking the end point.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.
5. SAMPLE PREPARATION

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For smoked fish and smoked fish products subject to compliance with section B.21.025 of the Food and Drug Regulations: remove the sample from the container with forceps or other suitable instrument, place on a coarse screen and allow to drain for ca 5 minutes. Place the sample on a paper towel or other clean absorbent paper and blot. Repeat blotting using a second and third piece of towel. Commence the sample until a homogeneous blend is obtained. If the sample texture is too tough for homogenization, pass the sample through a grinder a sufficient number of times to obtain a uniform mix. A food chopper may suffice as an initial step if the sample size is large.

5.1.2 For brined or salt-cured fish: scrape off as many loose salt crystals as possible with a spatula, if any are present, then wipe off any remaining salt crystals or moisture with a paper towel. Commence the sample until a homogeneous blend is obtained.

5.1.3 For raw fish, fresh or thawed: pass the sample through a grinder a sufficient number of times to obtain a homogenous blend.

5.1.4 For large fish: from each fish cut 3 cross-sectional slices of 2.5 cm (1 in.) from just back of pectoral fins, halfway between first slice and vent and just back of vent. Grind all pieces from all fish representing the sample until homogeneous.

5.1.5 For products that are packed in a medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Commence the part of the sample retained by the screen until a homogeneous blend is obtained.

5.1.6 For products that are packed in a medium that may be or is normally used by the consumer, transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.1.7 For processed products containing no separable liquid:
thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix. Thorough grinding and mixing is extremely important if the product is made up of several distinct components as in the case of fish dinners.

5.1.8 For fish meal: grind the sample in a mill or other suitable apparatus until it will pass through a no. 20 sieve.

5.1.9 For brine solutions: shake well before sampling.

5.1.10 For samples difficult to comminute or homogenize: dry the sample, determine the moisture content, and grind in the dry state to obtain homogeneity.

5.2 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. APPARATUS

6.1 Dessicator.

6.2 Hot plate, with variable temperature control.

6.3 Metal dish, ca 50 mm diameter and ca 40 mm deep, fitted with an inverted slip-on cover which fits tightly on the inside; or an equivalent container.

6.4 Tissue homogenizer or blender, with variable speed control.

6.5 Vacuum oven, with thermometer.

6.6 Vacuum pump, capable of maintaining partial vacuum in the oven equivalent to 100 mm of Hg, and equipped with a sulphuric acid gas-drying bottle.

7. REAGENTS

7.1 Ammonium thiocyanate (NH₄SCN), or potassium thiocyanate (KSCN).

7.1.1 Ammonium thiocyanate standard solution (0.1 N). Dissolve
7.613 g NH₄SCN in halogen-free water and dilute to 1 L. Standardize the solution as described in the Official Methods of Analysis of the A.O.A.C., 16th Edition (1995), method 942.26. If convenient, a 0.2 N solution or other concentration may be used.

7.2 Ferric alum (FeNH₄(SO₄)₂.12H₂O).

7.2.1 Ferric indicator. Prepare a saturated solution (45 %) of FeNH₄(SO₄)₂.12H₂O in halogen-free water.

7.3 Potassium chromate (K₂CrO₄).

7.3.1 Saturated, aqueous K₂CrO₄ solution.

7.4 Nitric acid (HNO₃).

7.5 Sulphuric acid (H₂SO₄).

7.6 Silver nitrate (AgNO₃).

7.6.1 Silver nitrate standard solution (0.1 N). Dissolve 16.99 g AgNO₃ in halogen-free water and dilute to 1 L. Standardize the solution as described in the Official Methods of Analysis of the A.O.A.C., 16th Edition (1995), method 941.18. If convenient, a 0.2 N solution or other concentrate may be used.

8. PROCEDURE

8.1 Determination of percent moisture

8.1.1 Dry the metal sample dish at 98 - 100 °C, cool in a dessicator, and weigh the dish (W1) soon after it reaches room temperature.

8.1.2 Accurately weigh (W2) an amount of the sample equal to ca 2 g dry weight in the tared dish.

8.1.3 Connect the vacuum pump and sulphuric acid gas-drying bottle to the vacuum oven. Loosely cover the dish, and dry the sample in the oven for 5 hrs at 95 - 100 °C under partial vacuum at 100 mm of Hg.

8.1.4 Admit dry air into the oven to bring it to atmospheric pressure. Immediately tighten the dish cover and transfer the fish to a dessicator.
8.1.5 Weigh the dish and contents (W3) soon after they reach room temperature. Dry the sample until constant weight results.

8.1.6 Determine the weight loss (WL) of the sample as follows:

\[ WL = W2 - (W3 - W1) \]

8.1.7 Determine the percent moisture of the sample as follows:

\[ \% \text{ Moisture} = \frac{WL}{W2} \times 100 \]

8.2 Determination of percent salt in fish and fish products

8.2.1 Accurately weigh 1 - 3 g of the prepared sample (from 5.2), depending upon the expected salt level, into a 250 mL erlenmeyer flask.

8.2.2 Pipet 25 - 50 mL of 0.1 N AgNO₃ into the flask, depending upon the expected salt level, add 20 mL conc. HNO₃ and boil gently on a hot plate in a fume hood until all solids except AgCl dissolve. (In the case of dyed products, it may be advantageous to boil the solution vigorously and reduce the volume to ca 20 mL to obtain a sharper titration end point.)

8.2.3 Add 50 mL of halogen-free water, cool to room temperature, add 3 mL of ferric indicator and titrate with 0.1 N NH₄SCN until the first persistent light brown color remains for approximately 15 seconds. Record the volume of 0.1 N NH₄SCN used in the titration.

8.3 Determination of percent salt in brine solutions

8.3.1 Accurately weigh 1 - 2 g of sample into an erlenmeyer flask and add ca 50 mL of chloride-free water. Titrate with 0.1 N AgNO₃ to a light brown end-point using a few drops of potassium chromate solution as an indicator.

9. CALCULATIONS

9.1 Calculate the percent salt in the sample as follows:

\[ \% \text{ NaCl} = \]

\[ \frac{[(\text{Vol. AgNO}_3 \times \text{N. AgNO}_3) - (\text{Vol. NH}_4\text{SCN} \times \text{N. NH}_4\text{SCN})] \times 58.44 \times 100}{\text{sample wt.} \times 1000} \]
9.2. To determine percent water-phase salt, multiply the percent NaCl (as calculated in 9.1) by 100 and divide by the percent moisture.

9.3 For brine solutions, calculate the percent salt as follows:

\[ \text{% NaCl} = \frac{\text{Vol. AgNO}_3 \text{ used} \times N. \text{AgNO}_3}{\text{sample wt. \times 1000}} \times 58.44 \times 100 \]

10. **PRECISION AND ACCURACY**

10.1 A relative standard deviation of less than 5 % and a relative error of less than 2 % can be expected with the argentometric method of salt determination.

11. **REMARKS**

11.1 It is important to be consistent in end-point recognition and the titration must be completed as quickly as possible to obtain accurate results. The use of a blank is essential.

11.2 For samples which contain a significant amount of pigmentation which may cause difficulty in end-point discrimination, potentiometric titration may be used if the necessary equipment is available; however, the mV change is small and not instantaneous. Therefore, it is not suitable for routine use and should only be used as an end-point indicator for difficult samples in conjunction with ferric indicator.

12. **REFERENCES**


CHAPTER 2 - PROXIMATE ANALYSIS  
SECTION 5: SODIUM AND POTASSIUM

1. SCOPE AND APPLICATION

1.1 This method is applicable to all fish and fish products. Depending upon the sensitivity required and the equipment available, either flame emission (F.E.) or atomic absorption (A.A.) can be used.

2. PRINCIPLE OF THE METHOD

2.1 The sample is dried at 100°C and mineralized in a muffle furnace at 525°C until a white ash is obtained. The ash is dissolved in dilute acid and the resulting sample solution is aspirated directly into the burner of either a flame emission (F.E.) or atomic absorption (A.A.) spectrophotometer, depending upon the sensitivity required and the equipment available.

3. INTERFERENCES

3.1 Ionization interferences are a problem with both the flame emission and atomic absorption methods.

3.1.1 For F.E., the Na and K standard solutions should contain the other element at the approximate level expected to be found in the diluted sample solutions. The emission intensity is also dependent upon the flame conditions.

3.1.2 For A.A., the presence of any ionizable metal will enhance the absorption of both Na and K, dependent upon flame conditions. The interferences can be minimized by adding a large excess of "the other element" (e.g. 1 or 2 mg/mL) to both the standards and sample solutions. The use of an air/hydrogen flame may also help to minimize the interferences.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.
5. **SAMPLE PREPARATION**

5.1 Take into account the type of product and how it is used and prepared by the consumer. Ensure that all the apparatus used is free from Na and K.

5.1.1 For products that are packed in a water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained. If the sample texture is too tough for homogenization, pass the sample through a grinder a sufficient number of times to obtain a uniform mix. A food chopper may suffice if the sample size is large.

5.1.2 For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.1.3 For products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix. Thorough grinding and mixing is extremely important if the product is made up of several distinct components as in the case of fish dinners.

5.2 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. (Some brands of disposable plastic cups have been proven to be free of Na and K and these may be used without previous cleaning.)

5.3 Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, reblend before use.

6. **APPARATUS**

6.1 Silica or platinum crucibles.

6.2 Muffle furnace.

6.3 Flame photometer, spectrophotometer or A.A. spectrophotometer.

6.4 Strip chart recorder; has advantages, but is not essential.

6.5 A diluter is very useful if a large number of samples with varying levels of Na and K are being analyzed.
7. **REAGENTS**

7.1 Nitric acid (HNO₃).

7.1.1 Nitric acid solution (1 + 4).

7.2 Sodium chloride (NaCl), 99.95% pure, heated at 500°C to constant weight and stored in a desiccator.

7.2.1 Sodium stock solution (1 mg/mL). Dissolve 2.5422 g of NaCl in distilled water and dilute to 1 L.

7.2.2 Sodium working standards. Prepare a set of 3 to 5 having a concentration range suitable for the instrument to be used and for the samples to be analyzed. Standards to be used for flame emission (for AA mode see 7.4) are as follows: 0.00, 0.01, 0.02, 0.03, 0.04, 0.05 mg/mL. Pipette 1, 2, 3, 4 and 5 mL of the stock Na solution into separate 100 mL volumetric flasks. To each add 7 mL of stock K solution, 2 mL conc HNO₃ and dilute to volume with distilled water. A blank should also be prepared in the same manner.

7.3 Potassium chloride (KCl), recrystallized, heated at 500°C to constant weight and stored in a desiccator.

7.3.1 Potassium stock solution (1 mg/mL). Dissolve 1.9068 g of KCl in distilled water and dilute to 1 L.

7.3.2 Potassium working standards. Prepare a set of 3 to 5 having concentrations suitable for the instrument to be used and for the samples to be analyzed. Standards to be used for flame emission (for AA mode see 7.4) are as follows: 0.00, 0.02, 0.04, 0.06, 0.08, 0.10 mg/mL. Pipette 0, 2, 4, 6, 8 and 10 mL of the stock K solution into separate 100 mL volumetric flasks. To each add 3 mL of stock Na solution, 2 mL conc HNO₃ and dilute to volume with distilled water. A blank should also be prepared in the same manner.

7.4 Working standards for the A.A. mode of measurement is in the range of 1 to 5 µg/mL and the level of the other element is about 1 or 2 mg/mL.

8. **PROCEDURE**

8.1 If an ash measurement has been carried out on the sample, using a silica crucible by a procedure such as A.O.A.C. 31.012, 12th edition, this ash may be used and step 8.2 can be omitted.
8.2 Accurately weigh ca 10.0 g of sample into a previously cleaned, acid-washed silica crucible. Place the weighed sample in a drying oven set at 110°C - 125°C for 8 to 24 hours. Transfer the dried sample into a muffle furnace set at 250°C. Gradually raise the temperature to 350°C over a period of 1 - 2 hours. Hold at this temperature until most of the fat is smoked off. Continue increasing the temperature to 525°C and hold for 8 to 16 hours. If by this time the ash is not white, remove from the muffle, cool, and add 1/4 to 1 mL of conc HNO₃ to the ash. Slowly evaporate off the HNO₃ on a hot plate (in a hood) until the ash is completely dry. Replace the crucible into the muffle for another 1/2 to 1 hour. The ash must be completely white. If it is not, repeat the HNO₃ acid treatment.

8.3 Dissolve the cooled ash with 15 mL dilute HNO₃ (1 + 4) on a warm hot plate. Filter the solution through a Whatman No. 42 paper into a 100 mL volumetric flask. Rinse the crucible and paper with 20 - 25 mL hot water followed by several cold water rinses to ensure a complete transfer of the soluble ash to the flask. (Omit the filtration step if the ash completely dissolves). Dilute the solution to volume with distilled water when cool.

8.4 Set up the photometer or spectrophotometer according to the manufacturer's instructions. Rotate the burner heat 90° and use an oxidizing flame. (Omit rotation of the burner head if additional sensitivity is required). Analyze the sample solution at 589 nm (Na) and 766 nm (K) by either F.E. or A.A. Dilute the sample solution to bring the emission or absorption reading within the range of the standard solutions. (Analyze the standard solutions frequently throughout a series of samples to check the calibration curve.) Alternatively, use the 330 nm (Na) and 404 nm (K) secondary wavelengths to reduce the number of dilutions required for the analysis of samples and standards with higher concentrations. Dilutions require 2 mL conc HNO₃ per 100 mL.

9. CALCULATIONS

9.1 Prepare calibration curves from the peak heights obtained for the standard solutions.

9.2 Determine the concentrations of Na and K in the sample by comparing the sample peak height to the calibration curve. Take into account the sample weight and any dilution factor.
10. REMARKS

10.1 Fish stored in refrigerated sea water (RSW) prior to landing and processing usually have increased sodium levels, accompanied by decreased potassium levels.

11. REFERENCES


CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 6: TOTAL REDUCING SUGARS

1. SCOPE AND APPLICATION

1.1 This method determines the total reducing sugars in prepared fish, calculated as dextrose, which contains filler, binder or other ingredients. The term "filler" includes flour or cereal meal prepared from grain or potato, starch, and added sugars.

2. PRINCIPLE OF THE METHOD

2.1 All the simple sugars and most of the disaccharide sugars possess the ability to reduce alkaline solutions of many metallic salts including copper. This phenomenon is utilized by the Fehling's Test for reducing sugars in which the sample solution is treated with a mixture of equal proportions of a copper sulphate solution and an alkaline tartrate solution. The Fehling's solution performs a two-fold purpose. It furnished a solution containing cupric ions that are reduced by the sugar to cuprous ions, and, in addition, the alkalinity is such that the sugar molecules are broken up into reactive fragments. These fragments are readily oxidized and cause the reduction of cupric ions to the cuprous state.

2.2 A weighed sample is hydrolysed to break down the carbohydrates into simple mono- and di-saccharides. The sample solution is treated with phosphotungstic acid to precipitate interfering substances, the extraneous solids are filtered from the solution, and an aliquot is treated with Fehling's solution to oxidize the reducing sugars. The cupric ions remaining in an aliquot of this solution are reduced with potassium iodide under acidic conditions, and a corresponding amount of iodide ion is oxidized to molecular iodine. The liberated iodine is titrated to a starch end-point with standard sodium thiosulphate solution, from which the original amount of reducing sugars can be calculated as dextrose.

3. INTERFERENCES

3.1 The presence of any materials capable of reducing cupric ions, or capable of oxidizing the reducing sugars, will interfere with the determination.
4. **SAMPLING PROCEDURE AND STORAGE**

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. **SAMPLE PREPARATION**

5.1 Comminute the sample until homogeneous, and place the homogenate in a thoroughly cleaned, sealable plastic or glass bottle. Store the sample in a refrigerator or freeze until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, reblend before use.

6. **APPARATUS**

6.1 Phosphoric acid flasks, 200 mL.

6.2 Tissue homogenizer, blender, or food grinder.

7. **REAGENTS**

7.1 Fehling's solution. The following solutions are not mixed prior to the determination since the mixture is not stable for any great length of time.

7.1.1 Copper sulphate solution. Dissolve 40 g of CuSO$_4$.5H$_2$O in distilled water and dilute to 1 L.

7.1.2 Rochelle salt solution. Dissolve 200 g of sodium potassium tartrate crystals in about 600 mL of hot distilled water, dissolve 150 g NaOH in the cooled solution and dilute to 1 L.

7.2 Hydrochloric acid (HCl).

7.2.1 Hydrochloric acid solution (1.5N). Dilute 130 mL of concentrated HCl to 1 L with distilled water.

7.2.2 Hydrochloric acid solution (1 + 2).

7.3 Sodium thiosulphate (Na$_2$S$_2$O$_3$.5H$_2$O).

7.3.1 Sodium thiosulphate solution (0.025N). Dissolve 6.5 g Na$_2$S$_2$O$_3$.5H$_2$O in distilled water and dilute to 1 L.
7.4 Potassium iodide (KI).

7.4.1 Potassium iodide solution (10%).

7.5 Sodium Hydroxide (NaOH).

7.5.1 Sodium hydroxide solution (20%).

7.6 Sulphuric acid solution (1 + 3).

7.7 Starch, powdered.

7.7.1 Starch indicator solution. Mix 1 g of powdered starch in 20 mL cold water and pour the mixture into 500 mL boiling water. Boil 10 minutes, cool and keep in a glass-stoppered bottle. Cover the solution with 1 mL of chloroform or toluene, or add 0.625 g salicylic acid as a preservative.

7.8 Phosphotungstic acid (P_2O_5.24WO_3.30H_2O, X - 48).

7.8.1 Phosphotungstic acid solution (20%). Filter if solution appears turbid.

7.9 Dextrose, anhydrous.

7.9.1 Standard dextrose solution. Dissolve 0.2 g in 100 mL distilled water.

7.10 Ammonium thiocyanate.

8. PROCEDURE

8.1 Weigh accurately 10.0 g of sample in a 200 mL phosphoric acid flask. Add 90 mL of 1.5N HCl, heat the flask for 90 minutes in a boiling water bath, and cool immediately to room temperature. Make the solution just alkaline to litmus (or phenolphthalein) with 20% NaOH solution (ca 27 mL), add 10 mL of HCl (1 + 2), and cool again if necessary.

8.2 Add 20 mL of 20% phosphotungstic acid solution, make to volume with distilled water, stopper and shake well. Allow the solution to stand 15-30 minutes and filter through 18.5 cm filter paper (Whatman No. 41 or No. 42, or equivalent).

8.3 Pipette 5 mL of filtrate into a 125 mL erlenmeyer flask, and add by pipette 5 mL of the copper sulphate solution and 5 mL of the alkaline Rochelle salt solution. Add 10-15 glass beads. Bring the solution to a boil on a hot plate and boil for approximately one minute, and then cool.
8.4 Add 20 mL of 10% potassium iodide solution and 4 mL of 
$\text{H}_2\text{SO}_4$ (1 + 3). Titrate with sodium thiosulphate solution 
adding 4 mL of starch indicator and ca 1 g of solid 
ammonium thiocyanate when the yellow iodine colour has 
almost disappeared. One drop of sodium thiosulphate should 
change the colour from blue to white or a faint lilac 
shade.

8.5 Run a blank determination using 5 mL of distilled water 
instead of the filtrate, starting at step 8.3. The 
difference between the blank and sample titrations will 
give the thiosulphate equivalent of the cereal (reducing 
sugars) present. Similarly, run a standard determination 
as above using 5 mL of the standard dextrose solution. 
Determine the blank and standard on the same day as the 
ample determination, and each time new solutions are used.

9. **CALCULATIONS**

9.1 The 5 mL of filtrate in the determination represent 0.25 g 
of the original sample $(10.0 \times 5)$. The 5 mL aliquot of 
the standard dextrose $200$ solution is 
equivalent to 10 mg of anhydrous dextrose $(0.4 \times 5)$. 
Therefore, an equivalent titration value to that obtained 
with the standard dextrose solution would represent 4% 
dextrose in the sample $(0.010 \times 100)$. 

9.2 The concentration of cereal (reducing sugars), calculated 
as dextrose, can be determined with the following formula:

$$\% \text{ Dextrose} = \frac{A - B}{A - C} \times 4$$

Where $A =$ blank titration (mL) 
$B =$ sample titration (mL) 
$C =$ standard dextrose titration (mL)

10. **PRECISION AND ACCURACY**

A collaborative study in 1986 between DFO inspection labs 
gave the following data for the precision of the method:
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<th>LAB NO.</th>
<th>MEAN (N=5)</th>
<th>S.D.</th>
<th>R.S.D.</th>
</tr>
</thead>
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<td>2A*</td>
<td></td>
<td>2.21</td>
<td>1.70</td>
<td>1.98</td>
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<td></td>
<td></td>
<td>2.12</td>
<td>1.81</td>
<td>1.77</td>
</tr>
<tr>
<td>2B</td>
<td></td>
<td>4.13</td>
<td>3.76</td>
<td>3.87</td>
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<tr>
<td></td>
<td></td>
<td>4.00</td>
<td>4.20</td>
<td>3.75</td>
</tr>
<tr>
<td>2C</td>
<td></td>
<td>0.00</td>
<td>0.32</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2.31</td>
<td>2.10</td>
<td>1.70</td>
</tr>
</tbody>
</table>

* 2A and 2D are the same sample.

11. REMARKS

11.1 The reducing sugars, after hydrolysis of all ingredients in the sample are actually treated as though they were dextrose and are reported as such.

11.2 For products containing more than 8% reducing sugars, use 2 mL of filtrate plus 3 mL distilled water in step 8.3. In this case the dextrose solution would represent 10% dextrose in the sample.

12. REFERENCES


12.4 Triebold, H.O.; and Aurand, L.W.; "Food Composition and Analysis", D. Van Nostrand Co. Inc. (1963), chapter 5.

CHAPTER 2 - PROXIMATE ANALYSIS
SECTION 7 : pH

1. SCOPE AND APPLICATION
1.1 This method is applicable to all fish and fish products.

2. PRINCIPLE OF THE METHOD
2.1 pH is a measure of the hydrogen ion concentration and is indicative of acidity. The pH of fish muscle is determined by measuring the electrical potential between glass membrane and reference electrodes against standard pH buffers.

3. INTERFERENCES
3.1 Formation of protein, oil or grease films on the electrodes may interfere with the measurement.

4. SAMPLING PROCEDURE AND STORAGE
4.1 Check and record sample information, including the condition of the sample, ensuring that fresh samples have been properly refrigerated and that frozen samples are still frozen.

4.2 Take a representative sample from the product lot and store so as to maintain sample integrity, taking into account the type of product and how it is stored commercially.

5. SAMPLE PREPARATION
5.1 Pass fresh, raw samples through a food blender a sufficient number of times to ensure a uniform mix and analyze immediately.

5.2 For products packed in a medium that may be or is normally used by the consumer, prepare a homogenous blend using the entire contents of the package.

5.3 For products packed in water, brine or a medium normally discarded by the consumer, drain the product on an appropriate size sieve for 1 - 1½ min. and prepare a homogeneous blend from the part of the sample retained by the sieve.
5.4 For processed products containing no separable liquid, thaw the product in the package (if frozen) and thoroughly comminute to obtain a homogeneous blend.

5.5 For pickled and semi-pickled fish, comminute the fish in the jar to obtain a homogeneous blend.

5.6 For samples containing sufficient oil to cause electrode fouling, separate the oil and blend the remainder to a homogeneous paste.

5.7 Collect the sample homogenates in thoroughly cleaned, sealable plastic cups and store in a refrigerator or freezer until analyzed.

6. **APPARATUS**

6.1 pH meter equipped with glass membrane indicator electrode and a calomel reference electrode (single or combination).

6.2 Blender or food processor.

6.3 Thermometer.

7. **REAGENTS**

7.1 Standard buffer solutions: commercial pH buffers 4, 6, 7 and 8.

7.2 Primary reference buffer solutions: NIST SRM (National Institute of Standards and Technology - Standard Reference Material) buffers against which working buffers are checked.

7.3 Reagent water: CO₂-free distilled or deionized water.

7.4 Saturated KCl solution.

8. **PROCEDURE**

8.1 Method A: with water addition

8.1.1 Switch on pH meter and allow electronic components to warm up and stabilize.

8.1.2 Equilibrate electrodes, buffers and samples at the same temperature.
8.1.3 Set temperature compensator control of instrument at the observed temperature.

8.1.4 Standardize pH meter according to manufacturer's instructions using NIST SRM buffers. Check working pH buffers 6 and 8 against the reference buffers.

8.1.5 Immerse electrodes in a 25 mL beaker containing the buffer solution, gently stirring the solution before taking readings. Between readings, store the electrodes in distilled or deionized water. Place the pH meter on "stand-by" mode when the electrode is removed from the solution. Blot (do not wipe) the electrode with a soft tissue.

8.1.6 Repeat standardization until the pH meter provides an accurate reading with the pH 7 working buffer.

8.1.7 Slurry 20 g of fish homogenate with 40 mL CO₂-free distilled water for 1 min. in a blender.

8.1.8 Pour some of the blend into a 50 mL beaker and check its temperature to ensure proximity to that of the buffers.

8.1.9 Immerse the electrodes in the slurry, ensuring good sample/electrode contact, before taking readings.

8.1.10 Take duplicate readings and report the results to 2 decimal places.

8.2 Method B: without water addition

8.2.1 Follow method A omitting step 8.1.7.

9. REMARKS

9.1 Keep calomel electrodes filled with saturated KCl solution.

9.2 Unless otherwise specified by the manufacturer, store glass electrode in pH 4 buffer, the reference electrodes in their own electrolyte-filling solution and the combination electrodes in pH 4 buffer containing a few drops of saturated KCl solution.

9.3 Some magnetic stirrers may affect pH readings; check during standardization.
10. REFERENCES


CHAPTER 3 - QUALITY INDICES
SECTION 1: HISTAMINE

1. SCOPE AND APPLICATION

1.1 "Histamine-like substances" are the principle compounds implicated as causing scombroid poisoning, an allergy-like condition caused predominantly by consumption of toxic fish of the sub-order Scombroidea which includes the tuna, bonito, kingfish, and mackerel. These compounds are formed as a result of growth of histidine decarboxylase-positive bacteria under conditions favourable for enzyme synthesis and activity. The level of "histamine-like substances" can be used as both an indicator of spoilage and as an indicator of substances of public health significance. This method is suitable for the analysis of "histamine-like substances" in the above species, fish products utilizing these species, and others like mahi-mahi (dolphinfish).

2. PRINCIPLE OF THE METHOD

2.1 The "histamine-like substances" are extracted from the sample with methanol, interfering compounds are removed by anion exchange chromatography, and the purified histamine is then derivitized with orthophthalaldehyde (OPT) to form a fluorophore. Its intensity is measured by fluorometry. The results are reported as equivalent histamine levels.

3. INTERFERENCES

3.1 In fish flesh, large amounts of the amino acid histidine may be present and this compound interferes with the determination of histamine, as it also forms a fluorophore with OPT. The anion exchange procedure removes histidine and its homologues. Several other polyamines may also react with OPT; however, they are normally present at low levels and are not a major problem.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

4.1.1 Refer to Sampling Plans 1 and 2 (AQL 6.5), Fish Product Standards and Methods Manual for number of sample units required for inspection.
5. **SAMPLE PREPARATION**

5.1 Upon receipt of the sample by the laboratory, the sample information should be checked and recorded in the laboratory records. The condition of the sample should be checked to ensure that fresh samples have been properly refrigerated and that frozen samples are still frozen. It is essential that a sample has been handled and stored in a manner that ensures its original quality has been maintained.

5.2 Fresh raw samples should be prepared immediately upon receipt but in no instance should a delay longer than 3 hours under refrigeration be permitted, otherwise the sample should be quickly frozen upon receipt. Store other samples to maintain their integrity, taking into account the type of product and how it is stored commercially.

5.3 Sample preparation should take into account the type of product and how it is used and prepared by the consumer. Samples that are too large or have a texture that is too tough for homogenization should be treated in a food processor or passed through a food grinder a sufficient number of times to ensure a uniform mix.

5.3.1 Fresh raw samples should be analyzed immediately after grinding but if this is not possible, they should be quick frozen to ensure that decomposition does not proceed.

5.3.2 Products packed in a medium that may be or is normally used by the consumer, e.g., fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.3.3 Products packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the sieve until a homogenous blend is obtained.

5.3.4 Processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a food grinder a sufficient number of times to obtain a uniform mix. Thorough grinding and mixing is extremely important if the product is made up of several distinct components e.g., fish dinners.
5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before analysis.

6. **APPARATUS**

6.1 Chromatographic columns, 150 x 9 mm i.d. or 200 x 7 mm i.d. glass or polypropylene or equivalent.

6.2 Volumetric flasks - 100 mL (Bates Sugar Flasks are easier to use than standard volumetrics).

6.3 Vortex mixer.

6.4 Fluorometer, having capability of excitation at 360 nm and emission at 450 nm.

6.5 Water bath set at 60 °C.

7. **REAGENTS**

7.1 All reagents are ACS grade unless specified otherwise. Distilled or deionized water is used throughout.

7.2 Methanol (CH$_3$OH).

7.2.1 Methanol (75%). Dilute 750 mL CH$_3$OH to 1 L.

7.3 Anion exchange resin - Bio-Rad AG1-X8, 50-100 mesh - if a substitute is used, its performance must be verified.

7.4 Hydrochloric acid (HCl).

7.4.1 Hydrochloric acid (1.0 N). Dilute 83 mL HCl to 1 L with distilled water.

7.4.2 Hydrochloric acid (0.1 N). Dilute 10 mL 1.0 N HCl to 100 mL with distilled water.

7.5 Sodium Hydroxide (97 % NaOH).

7.5.1 Sodium Hydroxide (1 N). Dissolve 41.2 g NaOH in distilled water and dilute to 1 L.
7.5.2 Sodium Hydroxide (2 N). Dissolve 82.4 g NaOH in distilled water and dilute to 1 L.

7.6 Phosphoric acid (85 % H₃PO₄).

7.6.1 Phosphoric acid (10 %). Dilute 12 mL of 85 % H₃PO₄ to 100 mL with distilled water.

7.7 Orthophthalaldehyde (O-phthalicdicarboxaldehyde) (C₆H₄(CHO)₂), (OPT).

7.7.1 Orthophthalaldehyde (OPT) solution (0.1 % w.v. in methanol). Store in dark bottle in refrigerator.

7.8 Histamine dihydrochloride (C₅H₉N₃.2HCl).

7.8.1 Histamine primary standard (1 mg/mL). Dry C₅H₉N₃.2HCl over sulphuric acid for 2 hours. Accurately weigh 0.165 g, dissolve, and dilute to 100 mL with 0.1 N HCl. Store in refrigerator.

7.8.2 Histamine standard (10 µg/mL). Dilute 1 mL of primary standard solution to 100 mL with 0.1 N HCl. Store in a refrigerator and prepare fresh every six months.

7.8.3 Histamine working standards (0.1, 0.2, 0.3 µg/mL). Dilute 1, 2, and 3 mL of 10 µg/mL standard to 100 mL with 0.1 N HCl. Store in a refrigerator and prepare monthly.

8. **PROCEDURE**

8.1 Convert the ion exchange resin to the - OH form by swirling it in a beaker with 2 N NaOH in the ratio of 15 mL per gram of resin. Let stand 20 to 30 minutes then decant the liquid and repeat the NaOH treatment. Thoroughly wash the resin with 2 or 3 similar volumes of H₂O, decanting after each rinse and then slurry it into a fluted filter paper such as S&S #588 or Whatman #2 using a wash bottle. Repeat the washing with water until the wash water is neutral to pH paper, then store the prepared resin under water.

8.1.1 Prepare the ion exchange column by placing a glass wool plug in the base of the column, then "slurry" in sufficient resin to form an 8 cm deep bed. Keep the resin covered with water at all times. Do not regenerate the resin in the column. Column conditioning can be done by applying a histamine standard solution (5 ppm in methanol) and washing
the resin with at least 10 mL of water (see section 11.2).

8.1.2 After each sample has been eluted, wash the resin with 10 mL of H₂O before applying the next sample. Approximately 50 samples may be run through each resin bed; however, a fewer number of samples with high salt content (e.g., anchovie) can be accommodated. Checks should be made to ensure that interfering compounds are not being eluted.

8.2 Accurately weigh 10.0 g of sample into a tissue homogenizer, add approximately 50 mL 75 % CH₃OH and blend approximately 2 minutes. Transfer the blend to a 100 mL volumetric flask. Rinse the homogenizer and lid well with 75 % CH₃OH and add the rinses to the volumetric flask. Place the flask in a hot-water bath heated to 60 °C and let stand at this temperature for 15 minutes. Cool to 25 °C and dilute to volume with 75 % CH₃OH then filter and store the filtrate in a refrigerator until it is to be analyzed. With canned products, filtering is usually not necessary and the clean supernatant may be decanted into a storage container.

8.3 To remove interferences from the extract, pass 4 or 5 mL of water through the ion exchange column and discard this solution. Pipette 1 mL of the extract onto the column and add 4 to 5 mL of water. Immediately initiate the column flow and collect it into a 50 mL volumetric flask which contains 5 mL of 1 N HCl. The flow rate is not critical and high flow rates can be used. When the liquid level is approximately 2 mm above the top of the resin, add a 2nd 5 mL portion of water followed by increasingly large volumes of water until a total of approximately 35 mL has been eluted. Stop the flow and dilute to 50 mL with water and mix. Store the eluate in a refrigerator until it is to be analyzed. Such extracts are stable for several weeks.

8.4 Reaction to prepare fluorophore: the timing is important; normally 10 or more OPT reactions can be run simultaneously by adding reagents in set order. Volumes may be altered to make use of available glassware as long as proportions remain constant and fluorescent output is within the measurement capability of the fluorometer.

8.4.1 Prepare blank and standard solutions by pipetting into the same size glass or plastic containers (as used for the reaction) 5 mL 0.1 N HCl, 5 mL of 0.1, 0.2, and 0.3 µg/mL
histamine working standards and 10 mL of 0.1 N HCl. Add 3 mL of 1 N NaOH, thoroughly mix. Within 5 minutes add 1.0 mL of 0.1 % OPT solution, thoroughly mix and wait exactly 4 minutes. Add 3 mL of 10 % H3PO4 solution and mix thoroughly. Prepare sample solutions using 5 mL of the column eluate and proceed as above. A check sample with a known histamine content should be run with each series of samples.

8.4.2 Within 1.5 hr, read the fluorescence of each solution using a sensitivity setting on the fluorometer which will give approximately 80 % full-scale reading with the 0.3 µg/mL standard.

8.4.3 To check for the approximate necessary dilution of samples with an off-scale fluorescent reading, two methods may be used: if the fluorometer has various sensitivity ranges, the sample may be read on a less sensitive range. Otherwise, the developed sample solution may be diluted with some of the developed blank solution and the fluorescence of the mixture read. To obtain an accurate result, the sample eluate should be diluted with 0.1 N HCl and a reaction run on this diluted eluate.

9. CALCULATIONS

9.1 Prepare a calibration curve of the fluorescence values versus the concentration of the histamine in the working standards.

9.2 Compare the sample fluorescence with the calibration curve and determine the concentration of histamine in the sample extract. Calculate the mg histamine free base per 100 g of sample as follows:

\[
\text{mg Histamine/100 g fish} = (10)(F)(I/m)(I_a) \\
\text{µg Histamine/g fish} = 10 \times (\text{mg histamine/100g})
\]

where \(I_a\), \(I_s\), \(I_b\), and \(I_c\) = fluorescence from sample, 0.1, 0.2, 0.3 µg histamine standards, respectively; and \(F = \text{dilution factor} = (\text{mL eluate} + \text{mL 0.1 N HCl})/\text{mL eluate}\). \(F = 1\) for undiluted eluate. If the calibration plot is not
linear, use standard curve directly for quantitation. Each subdivision on the abscissa should be \(\leq 0.1 \mu g\) histamine/5 mL solution. Read all values from curve to nearest 0.05 \(\mu g\) histamine/5 mL solution.

\[
\begin{align*}
\text{mg Histamine/100 g fish} &= (10)(F)(W) \\
\text{\(\mu g\) Histamine/g fish} &= 10 \times (\text{mg histamine/100g})
\end{align*}
\]

where \(W = \mu g\) histamine/5 mL solution as determined from standard curve.

If all dilutions are as specified in this procedure, the 0.2 \(\mu g/mL\) standard is equal to 10 \(\mu g/mL\) in the methanol extract or to 10 mg % histamine in a sample.

10. PRECISION AND ACCURACY (preliminary data)

10.1 All parameters in the fluorophore reaction have been tested for ruggedness. The only feature that must be carefully controlled and be uniform is the timing of the reaction. The given times \(\pm \frac{1}{4}\) minute give the highest fluorescence reading and best reproducibility. The volumes and strength of the NaOH, OPT and \(H_3PO_4\) have fairly wide latitudes and those given \(\pm 20\%\) give reproducible results.

10.2 To test the repeatability for the elution of histamine from the column and the possible life of a column, a 5.0 mg % standard histamine solution and an extract from tuna having a mean histamine level of 14.4 mg % were run repeatedly through the same newly poured column over a period of about one month. With the histamine standard solution, the standard deviation was less than 0.2 mg % and the results ranged from 4.2 to 5.2 mg %. With the tuna extract the standard deviation was 0.3 mg % and the range 13.3 to 15.0. It was noted during this experiment that the first 6 to 8 samples through the new column all give lower results. Seventy samples were run through each column and the results were still consistent.

10.3 To check for percent recovery of added histamine, samples of good and decomposed tuna were spiked with histamine at various levels, thoroughly mixed and permitted to stand in a refrigerator about one week before analysis. Recovery from the good tuna was approximately 95 % and from the decomposed tuna 101 %. The standard addition results for the unspiked samples were the same as the analytical
results.

11. REMARKS

11.1 Experimentation has shown that samples with a high histamine content will influence the results of succeeding samples. For example, after a sample containing 10 mg % histamine is passed through a column, the following sample passing through the same column tends to have a reading approximately 0.3 mg % higher than actual.

In order to determine whether or not a column is still providing acceptable results, it is recommended that a sample containing a known histamine value be analyzed along with each run. From time to time, it is also a good idea to analyze a sample not containing any histamine.

11.2 While the AOAC method does not require putting standards through the column, it is another option for checking on the acceptability of a column. This can be done by replacing the 1 mL of extract in the procedure described in 8.3 with 1 mL of a methanol working standard and carrying the standard solution through procedures described in 8.3 to 8.4.2. The standards in methanol are prepared as follows:

a) Histamine standard in methanol (100 µg/mL)

Dilute 10 mL of histamine primary standard to 100 mL with methanol. Store in refrigerator.

b) Histamine working standards in methanol (5, 10, and 15 µg/mL)

Into separate 100 mL volumetric flasks, pipette 5, 10, and 15 mL of the histamine methanol standard and dilute to 100 mL with methanol. These standards are stable for at least 2 weeks stored in a refrigerator.

The fluorescence from the 3 methanol standards should be the same as obtained with 3 working standards in HCl. If the recovery of histamine is not at least 95 %, a second set of standards should be run through the columns and if no improvement is then observed, the resin should be re-prepared and the columns repoured. Normally the percent recovery should be 97-99 %.
12. REFERENCES


CHAPTER 3 - QUALITY INDICES
SECTION 2: TRIMETHYLAMINE NITROGEN

1. SCOPE AND APPLICATION

1.1 Trimethylamine (TMA) nitrogen value is applicable to certain marine species and is a useful indicator of loss of freshness through slow aerobic spoilage, such as would occur at the temperature of melting fresh water ice.

2. PRINCIPLE OF THE METHOD

2.1 The fish tissue sample is extracted with an aqueous solution of trichloroacetic acid. Formalin is added to an aliquot of the extract to remove the ammonia present by reacting with it to form hexamethylene-tetramine, alkali is added and the TMA free base is extracted into toluene. Picric acid is added to the toluene extract to complex with the TMA, and the absorbance of the solution is measured spectrophotometrically at 410 nm.

2.2 The spoilage of fish stored in ice is due to bacterial and enzymatic action which results in the production of various volatile compounds, in particular trimethylamine, dimethylamine, ammonia and volatile acids. Trimethylamine oxide, a constituent of marine fish, is reduced during spoilage to TMA, and the ammonia formed is mainly a product of protein breakdown.

3. INTERFERENCES

3.1 Traces of water in the toluene extract will interfere with the TMA/picric acid reaction.

3.2 Dimethylamine, which forms mainly in frozen storage, will interfere with the TMA assay.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.
5. SAMPLE PREPARATION

5.1 The condition of the sample should be checked to ensure that fresh samples have been properly refrigerated and that frozen samples are still frozen. It is essential that a sample has been handled and stored in a manner that ensures its original quality has been maintained.

5.2 Fresh raw samples should be prepared immediately upon receipt but in no instance should a delay longer than 3 hours under refrigeration be permitted, otherwise the sample should be quick frozen upon receipt.

5.3 Samples that are too large or have a texture that is too tough for homogenization should be treated in a food processor or passed through a food grinder a sufficient number of times to ensure a uniform mix.

5.3.1 Fresh raw samples should be analysed immediately after grinding but if this is impossible, they should be quick frozen to ensure that decomposition does not proceed.

5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before analysis.

6. APPARATUS

6.1 Centrifuge.

6.2 Centrifuge tubes, 50 mL.

6.3 Timer.

6.4 Spectrometer.

7. REAGENTS

7.1 Magnesium carbonate (MgCO₃).

7.2 Potassium carbonate (K₂CO₃).

7.2.1 Potassium carbonate solution. Dissolve 100 g K₂CO₃ in 100 mL distilled water.
7.3 Sodium sulphate (Na$_2$SO$_4$), anhydrous.

7.4 Sulphuric acid (H$_2$SO$_4$).

7.4.1 Sulphuric acid solution (1N). Dilute 28.35 mL conc H$_2$SO$_4$ to 1L with distilled water.

7.5 Toluene, dried over anhydrous Na$_2$SO$_4$.

7.5.1 To remove interferences, shake 500 mL toluene with 100 mL 1N H$_2$SO$_4$, distill and dry over anhydrous Na$_2$SO$_4$.

7.6 Trichloroacetic acid (TCA).

7.6.1 TCA solution (7.5%, aqueous).

7.7 Formalin (HCHO), available commercially as 40% solution.

7.7.1 Formalin solution (20%). Shake 1L 40% formalin with 100 g MgCO$_3$ until nearly colourless and filter. Dilute 100 mL of filtrate to 200 mL with distilled water.

7.8 Picric acid.

7.8.1 Picric acid stock solution. Dissolve 2 g picric acid in 100 mL water-free toluene.

7.8.2 Picric acid working solution. Dilute 1 mL stock solution to 100 mL with water-free toluene.

7.9 Trimethylamine hydrochloride (TMA.HCl).

7.9.1 TMA stock standard solution. Add 0.682 g TMA.HCl to 1 mL HCl (1 + 3) and dilute to 100 mL with distilled water. Contains ca 1 mg TMA.N/mL and must be checked by micro-Kjeldahl method.

7.9.2 TMA working standard solution (0.01 mg TMA.N/mL). Add 1 mL stock standard solution to 1 mL HCl (1 + 3) and dilute to 100 mL with distilled water.
8. **PROCEDURE**

8.1 Accurately weigh 100 g of sample into a blender, add 200 mL of 7.5% TCA and homogenize for 2 minutes. Centrifuge an aliquot of this extract at 2000 - 3000 rpm until the supernatant is practically clear.

8.2 Pipet an aliquot of the TCA extract containing 0.01 - 0.03 mg TMA.N into a 20 x 150 mm Pyrex screw-cap test tube and dilute to 4.0 mL with distilled water.

8.3 Prepare a reagent blank and calibration standards by pipetting 0, 1.0, 2.0, and 3.0 mL of TMA working standard solution into screw-cap test tubes and diluting to 4.0 mL with distilled water.

8.4 To the sample, blank and standards, add sequentially 1 mL 20% formalin solution, 10 mL toluene, and 3 mL K₂CO₃ solution. Cap the tubes and place on a tube rotator for 12 minutes or shake vigorously by hand approximately 40 times.

8.5 Transfer 7-9 mL of the toluene layer to a small drying tube containing ca 0.1 g anhydrous Na₂SO₄. Avoid transferring any droplets of the aqueous phase. Stopper the tube and shake well to dry the toluene extract.

8.6 Pipet 5 mL of the toluene extract (from the drying tube) into a dry cuvette, add 5 mL picric acid solution and gently swirl to mix.

8.7 Set up the spectrometer as specified by the manufacturer, and measure the absorbances of the sample and standards relative to the reagent blank at 410 mm. (The TMA/picric acid complex is stable.)

9. **CALCULATIONS**

9.1 Calculate the mg TMA.N/100 g of sample (based on a 1 mL aliquot used in step 8.2) as follows:

\[
\text{mg TMA.N/100 g} = \frac{A \times C' \times V'}{A'} \times 300
\]

where

- \( A \) = absorbance of the sample solution
- \( A' \) = absorbance of the standard solution nearest to the absorbance of the sample
- \( C' \) = concentration of the standard solution used (in mg TMA.N/mL)
- \( V' \) = volume (mL) of the standard solution used
10. **PRECISION AND ACCURACY**

10.1 Duplicate analyses usually agree to within 5%.

11. **REMARKS**

11.1 Do not use stopcock grease. Mixture of sugar and glycerol ground together may be used if necessary.

11.2 Do not wash tubes with soap or detergent. Rinse with distilled water and occasionally clean with concentrated HNO$_3$.

11.3 Trimethylamine nitrogen values for the three grades of haddock, cod and American plaice were found to be:

<table>
<thead>
<tr>
<th></th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haddock &amp; Cod</td>
<td>0.00 to 1.19</td>
<td>1.20 to 5.29</td>
<td>more than 5.29</td>
</tr>
<tr>
<td>American Plaice</td>
<td>0.00 to 1.09</td>
<td>1.10 to 4.59</td>
<td>more than 4.59</td>
</tr>
</tbody>
</table>

Grade 1 = Top Quality  
Grade 2 = Second Quality  
Grade 3 = Reject Quality

12. **REFERENCES**


CHAPTER 3 - QUALITY INDICES
SECTION 3: PUTRESCINE AND CADAVERINE

1. SCOPE AND APPLICATION

1.1 The diamines putrescine and cadaverine, which are formed from the amino acids ornithine and lysine, are the products of bacterial decomposition of fish tissue. These diamines can be used as indices of decomposition in fish and shellfish.

2. PRINCIPLE OF THE METHOD

2.1 Putrescine and cadaverine are extracted with methanol, an internal standard hexanediamine is added, and a dry residue of their hydrochloride salts is prepared. The salts are derivitized with pentafluoropropionic anhydride, and then separated from excess reagent on an alumina column. The derivatives are then injected into a gas chromatograph with an electron capture detector to determine the levels of putrescine and cadaverine.

3. INTERFERENCES

3.1 There may be extraneous peaks which may interfere with the integration.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Upon receipt of the sample by the laboratory, the sample information should be checked and recorded in the laboratory records. The condition of the sample should be checked to ensure that fresh samples have been properly refrigerated and that frozen samples are still frozen. It is essential that a sample has been handled and stored in a manner that ensures its original quality has been maintained.
5.2 Fresh raw samples should be prepared immediately upon receipt but in no instance should a delay longer than 3 hours under refrigeration be permitted, otherwise the sample should be quick frozen upon receipt. Store other samples to maintain their integrity taking into account the type of product and how it is stored commercially.

5.3 Sample preparation should take into account the type of product and how it is used and prepared by the consumer. Samples that are too large or have a texture that is too tough for homogenization should be comminuted in a food processor or passed through a food grinder a sufficient number of times to ensure a uniform mix.

5.3.1 Fresh raw samples should be analysed immediately after grinding; but if this is impossible, they should be quick frozen to halt decomposition.

5.3.2 Products packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.3.3 Products packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the sieve until a homogeneous blend is obtained.

5.3.4 Processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a food grinder a sufficient number of times to obtain a uniform mix. In the case of products that are made up of several separate distinct components such as fish dinners, only the fish component would be analyzed.

5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before analysis.
6. APPARATUS

6.1 Volumetric flasks - 100 mL (Bates sugar flasks are easier to use than standard volumetrics).

6.2 Chromatographic columns 150 x 9 mm I.D. glass with reservoir.

6.3 Round bottom flasks and stoppers 100 mL (250 mL are usable) or flat bottom boiling flasks, 100 - 125 mL with 24/40 T$ stoppers.

6.4 Culture tubes 150 x 25 mm with screw tops or 60 mL bottles with screw tops or equivalent.

6.5 Gas chromatograph with electron capture detector.

6.6 Column for gas chromatograph - glass column 1.8 x 4 mm packed with 3% OV-225 on 100-120 mesh Supelcoport or equivalent.

6.7 Polytron mixer.

6.8 Tall form beakers - 200 mL; or erlenmeyer flasks - 125 mL.

6.9 Filter paper - general purpose, fast filtering type.

7. REAGENTS

7.1 Methanol (CH$_3$OH) ACS Grade.

7.2 Hydrochloric acid (HCl).

7.2.1 Hydrochloric acid (1.0 N): dilute 82.4 mL HCl to 1 L with distilled water.

7.2.2 Hydrochloric acid (0.1 N): dilute 8.24 mL HCl to 1 L with distilled water.

7.3 Hexanediamine.

7.3.1 Hexanediamine stock solution (1 mg/mL): dissolve 100 mg hexanediamine in 0.1 N HCl and make up to 100 mL in a volumetric flask.

7.3.2 Hexanediamine working solution (10 ug/mL): dilute 1 mL stock solution to 100 mL in a volumetric flask with 0.1 N HCl; used as an internal standard.
7.4 Putrescine (1, 4 - diaminobutane).

7.5 Cadaverine (1, 5 - diaminopentane).

7.6 Standard diamine stock solution (1 mg/mL cadaverine and putrescine): dissolve 100 mg putrescine and 100 mg cadaverine in 0.1 N HCl and make up to 100 mL in a volumetric flask.

7.6.1 Standard diamine working solution A (10 ug/mL): dilute 1 mL stock solution to 100 mL in a volumetric flask with 0.1 N HCl.

7.6.2 Standard diamine working solution B (1 ug/mL): dilute 10 mL working solution A to 100 mL in a volumetric flask with 0.1 N HCl.

7.7 Pentafluoropropionionic anhydride (PFPA).

7.8 Ethyl acetate (glass distilled).

7.9 Hexane (glass distilled).

7.10 Toluene (glass distilled).

7.11 Alumina (activated neutral 150 mesh).

7.11.1 Alumina (3% moisture)
Heat alumina in oven at 125°C for 2 hours. Cool in dessicator. Add 3.0 mL distilled water to 97.0 g alumina, stopper and shake until homogeneous; allow to set for 5 hours for equilibration.

7.12 Sodium sulphate - granular anhydrous.

8. PROCEDURE

8.1 Accurately weigh 10.0 g of sample into a 200 ml tall form beaker or 125 mL erlenmeyer flask. Add approximately 60 ml methanol and blend until homogeneous using a polytron mixer (approximately 1 min). Transfer (rinsing with methanol) to a 100 mL volumetric flask. Make up to volume with methanol and mix thoroughly.
8.2 Allow the sample residue to settle until a clear extract is obtained. Alternatively the extract may be filtered or centrifuged to obtain a clear solution. Store the extract in a refrigerator until it is to be analysed.

8.3 For standards:

Pipet diamine working solutions as follows into 100 mL round bottomed or flat bottomed boiling flasks.

<table>
<thead>
<tr>
<th>Working solution</th>
<th>mL Used</th>
<th>Equivalent diamine in sample ug/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

If an integrator is being used that only allows one standard, use the 1 ug/g standard.

For Samples:

Pipet 10 mL of clear extract into 100 mL round bottomed or flat bottomed boiling flasks.

8.4 To a round bottomed or flat bottomed boiling flask (containing the standard or sample extract) pipet 1 mL hexanediamine working solution (internal standard) and 0.5 ml 1N HCl. Evaporate to dryness on a rotary evaporator at 50° C.

Any water present in the sample will hinder derivatization. The sample should be completely dry prior to commencing step 8.4.

8.5 To react the residue, add 1 mL ethyl acetate and 500 uL pentafluoropropionic anhydride, stopper, mix and heat in a water bath at 50° C for 30 minutes. Swirl solution at least once during the reaction. Ensure the stoppers are loosely seated when the flasks are put into the water bath and recheck occasionally.
If the reaction has not cleared within 15 minutes an additional 300 uL should be added. Failure to obtain a clear (usually yellow) reaction mixture means the reaction has not proceeded properly, i.e. if milky or cloudy - no reaction.

8.6 After reaction, evaporate the solvent and the excess reagent under nitrogen at 50°C. Dissolve the residue in 2 mL of 30% ethyl acetate in toluene (if needed this can be left at 4°C overnight).

8.7 Prepare an alumina column by putting in a piece of glasswool and filling a chromatographic column with alumina to a height of 8 cm. Cover the alumina with a layer (1 cm) of anhydrous sodium sulphate.

8.8 Add 10 mL hexane to the alumina column. Collect the column effluent in a screw top test tube or bottle. When the hexane level reaches the surface of the alumina column, add the extract. Rinse the flask with 3 mL 30% ethyl acetate in toluene and add to column in a similar manner. Repeat with 18 mL of 30% ethyl acetate in toluene. Collect all the column effluent, place the screw cap on test tube and mix. The effluent is quite stable at room temperature for at least several days.

8.9 Gas chromatograph conditions are as follows:

<table>
<thead>
<tr>
<th></th>
<th>180 ° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td></td>
</tr>
<tr>
<td>Injector port</td>
<td>200 ° C</td>
</tr>
<tr>
<td>Detector</td>
<td>320 ° C</td>
</tr>
<tr>
<td>Carrier gas flow nitrogen</td>
<td>60 mL/min</td>
</tr>
</tbody>
</table>

8.10 Inject 1 or 2 uL into the gas chromatograph. Approximate retention times are, putrescine 6.4 min, cadaverine 8.6 min, and hexanediamine 10.6 min.

9. CALCULATIONS

9.1 If an integrator - data handling system, is not used, calculate the ratio of the standard peak height (putrescine or cadaverine) to the peak height of the hexanediamine. From the standard ratios, plot a calibration curve (this should be linear from 1 ug/g to 20 ug/g). The concentration of putrescine and cadaverine can then be calculated.
Example:

\[
\text{Cpu} = \frac{\text{Cps} \times \text{Hiss} \times \text{Hpu}}{\text{Hps} \times \text{Hisu}}
\]

pu = putrescine in unknown
ps = putrescine in standard
iss = internal standard in standard
isu = internal standard in unknown

10. **PRECISION AND ACCURACY**

10.1 Preliminary data (DFO check sample studies) indicates the reproducibility is \(\pm 0.1\) ug/g at 1.0 ug/g level and \(\pm 0.3\) ug/g at the 10.0 ug/g level.

11. **REMARKS**

11.1 Experimental data indicates the recovery of cadaverine is 95% or better and putrescine at 80-90%.

11.2 With some products (most often spoiled uncooked samples) there are sometimes extraneous peaks which may interfere with the integration of putrescine and cadaverine.

11.3 Sometimes a yellow colour will elute from the alumina column. *In general the sample may still be used; however it may interfere with the putrescine/cadaverine quantification. In these cases the sample should be re-extracted.*

12. **REFERENCES**


CHAPTER 4 - FOOD ADDITIVES
SECTION 1: SULPHITE

1. SCOPE AND APPLICATION

1.1 This method is applicable to fresh and frozen fish and shellfish that do not contain high levels of interfering compounds.

2. PRINCIPLE OF THE METHOD

2.1 Sulphur dioxide (SO₂) is liberated by acidifying the sample with HCl and boiling under reflux in a nitrogen (N₂) atmosphere. The liberated SO₂ is steam-distilled into a 3 % hydrogen peroxide (H₂O₂) solution, which oxidizes the SO₂ into H₂SO₄, which is titrated with a standard NaOH solution.

2.2 The presence of SO₂ may be confirmed by gravimetric analysis of the final solution from 2.1. The sulphate is precipitated with BaCl₂ solution. The precipitated BaSO₄ is collected by filtration, weighed, and the amount of SO₂ present is calculated.

3. INTERFERENCES

3.1 In 2.1, other free acids and acids that are liberated by HCl can be distilled over and titrated as SO₂. Hydrogen peroxide solutions can also contain traces of H₂SO₄ and it is therefore necessary to run blanks.

3.2 The gravimetric procedure is accurate but quite sensitive to operator error. Care must be taken to ensure that each Gooch crucible is properly prepared and dried, and that the precipitate is thoroughly washed and carefully dried.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

4.2 Samples should be frozen in an air-tight container prior to preparation and analysis.
5. **SAMPLE PREPARATION**

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For products that are packed in a water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained. If the sample texture is too tough for homogenization, pass the sample through a grinder a sufficient number of times to obtain a uniform mix. A food chopper may suffice if the sample size is large.

5.1.2 For products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix.

5.1.3 For glazed frozen flesh: deglaze and discard the meltwater, then prepare the sample as described in 5.1.2.

5.1.4 For frozen shell-on products: deglaze and remove the head, shell, etc., then prepare the sample as described in 5.1.2.

5.1.5 For dried product: test sample in the dried form.

5.2 Collect the homogenized sample into a clean, sealable plastic cup or glass bottle.

5.3 Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, reblend before use.

6. **APPARATUS**

6.1 Modified Monier-Williams apparatus (see Figure 1).

6.2 Heating mantle (for 1 L flask), with variable transformer.

6.3 Nitrogen cylinder (Oxygen-free Grade), with two-stage regulator.

7. **REAGENTS**
7.1 All reagents are ACS grade unless specified otherwise. Distilled or deionized water is used throughout.

7.2 Hydrogen peroxide (H$_2$O$_2$, 30 %).

7.2.1 Hydrogen peroxide solution (3 %). Dilute 30 % H$_2$O$_2$, 1 to 10.

7.3 Hydrochloric acid (HCl), conc.

7.3.1 HCl solution (1 + 2).

7.3.2 HCl solution (6 N). Dilute 534 mL conc. HCl to 1 L with distilled water.


7.5 Barium chloride (BaCl$_2$).

7.5.1 Barium chloride solution (10 %). Filter before use.

7.6 Neutral methyl red indicator (0.25 % in alcohol).

7.7 Ethyl Alcohol (95 %).

7.8 Ethyl Ether.

7.9 Pyrogallol.

7.10 Potassium Hydroxide (KOH).

8. PROCEDURE

8.1 If Oxygen-Free Grade nitrogen (< 5 ppm O$_2$) is not used, the nitrogen gas must be purified by the following procedure:

Grind 4.5 g of pyrogallol with 5 mL of water and transfer the slurry to the gas-washing bottle of the Monier-Williams apparatus. Repeat the grinding and transfer with two additional 5 mL water portions. Connect a nitrogen cylinder equipped with a two-stage regulator to the gas inlet tube and flush the air out of the gas-washing bottle. Add a cooled solution of 65 g of KOH dissolved in approximately 85 mL of water to the gas-washing bottle
through a long-stemmed funnel. Turn off the nitrogen and connect the gas-washing bottle to the distillation flask with a short piece of acid-washed silicone rubber tubing. Clamp off both ends of the gas-washing bottle. Prepare the gas-washing solution fresh daily.

8.2 Assemble the remainder of the Monier-Williams apparatus (using acid-washed silicone tubing for the connections where necessary) and place the heating mantle under the distillation flask. Add to the exit side of each U-tube two ca 2 cm lengths of 25 mm diameter solid glass rod, 10 mL of 3 mm glass beads and 10 mL of 3 % H₂O₂ solution containing one drop of methyl red indicator. The bottom of the U-tube must be completely filled with glass beads and liquid.

8.2.1 Attach a piece of rubber tubing to the separatory funnel, open the funnel stopcock, blow lightly into the tubing, close the funnel stopcock and check for gas leaks in the apparatus by waiting a few minutes then noting any change in liquid level in the U-tubes.

8.3 Remove the separatory funnel from the distillation flask and transfer into the flask an accurately weighed sample, e.g., 50 g or an amount estimated to contain more than 45 mg of SO₂, using water if necessary to complete the transfer. (The sample may be weighed into the flask before the apparatus is assembled in 8.2.)

8.3.1 Dilute the sample to ca 400 mL with water. Replace the separatory funnel, close the stopcock, and pour 90 mL HCl (1+2) into the funnel. Force the HCl into the distillation flask using gentle pressure. Start a gentle flow of N₂ and carefully heat the flask so that the solution will begin to reflux in 20 to 25 minutes. Apply full line voltage to the heating mantle and reflux the solution for 1½ hours.

8.3.2 Turn off the water to the condenser and continue heating until the first joint of the first U-tube shows condensation and becomes warm. Remove the funnel and turn off the heat and N₂ flow. When the top of the condenser is cool, remove the connecting assembly and rinse into the first U-tube. Rinse the cross-over tube into the second U-tube.

8.3.3 Add a drop of methyl red indicator to the first U-tube and titrate the solution with 0.1 N NaOH, gently rocking the U-
tube to mix solution. Save the titrated solution for confirmatory analysis.

8.3.4 Titrate the second U-tube similarly. Transfer the solutions of both U-tubes quantitatively into a 400 mL beaker, using a sieve for collecting and washing the glass rods and beads. Total volume after the transfer should not exceed 250 mL.

8.4 Gravimetric Confirmation: Add 5 mL of 6 N HCl (for 250 mL of solution) to the beaker and heat to boiling. Slowly add filtered 10% BaCl₂ solution with stirring until precipitation is complete and add 2 mL in excess. Add at least 10 mL BaCl₂ solution if the amount of precipitate is small. Cover the beaker and digest the solution at 80-90 °C for at least 2 hours, preferably overnight.

8.4.1 Decant the solution and precipitate through a previously prepared, dried and weighed Gooch crucible. Wash the beaker and precipitate thoroughly with hot water (5 to 8 washes), transferring all the precipitate to the crucible. Test the last wash for absence of chloride by adding a few drops of AgNO₃ solution. If a precipitate forms, continue washing until the wash solution is free from chloride. Wash the precipitate with 20 mL of ethyl alcohol followed by 20 mL of ethyl ether. Dry the crucible to constant weight at 105-110 °C and record the weight.

8.4.2 If Gooch crucibles are not available, use ashless filter paper and ordinary porcelain crucibles. Pre-ignite the crucibles at approximately 800 °C, cool and weigh prior to use. Collect the precipitate by filtering through the ashless filter paper. Place the filter and precipitate into the crucible and ignite the filter paper at approximately 800 °C. Keep the crucible covered to avoid having the paper burst into flame.

8.5 Determine blanks on the reagents for both the titration and gravimetric procedures.

9. Calculations

9.1 Titration procedure: Calculate the volume of standard NaOH required by adding together the titres required for each U-tube and subtracting the titre required for the reagent blank. Calculate the SO₂ present as follows:
ppm SO₂ = \frac{vol. (mL) of 0.1 \text{ N NaOH \times 10}^3 \times 3.203}{weight (g) of sample}

9.2 Gravimetric procedure: Correct the weight of the sample precipitate by subtracting the weight of the reagent blank. Calculate the SO₂ present as follows:

ppm SO₂ = \frac{weight (mg) \text{ BaSO₄ \times 274.46}}{weight (g) of sample}

10. PRECISION AND ACCURACY

10.1 Titration procedure: Sulphite values of less than 20 ppm are considered as insignificant due to possible interference. Values of 20 ppm and over are indicative of presence of SO₂. Confirmation by the gravimetric procedure is desirable.

10.2 Gravimetric procedure: An accuracy of ± 1 ppm is possible when a 50 g sample is used.

11. REMARKS

11.1 When analyzing fish products to determine if sulphite has been added as a preservative, it must be recognized that it is possible for the thio-proteins to be degraded to produce SO₂ in the presence of HCl. If levels of SO₂ between 10 and 20 ppm are obtained, the results should only be presumptive evidence that sulphite was added to the product.

12. REFERENCES

FIGURE 1:
MODIFIED MONIER–WILLIAMS APPARATUS

A. – Gas Washing Bottle, 250 mL.
B. – Gas Inlet Tube, $24/40$ joint.
C. – Separatory Funnel, 125 mL $24/40$ joint.
D. – Heating Mantle, 1L size.
E. – Distillation Flask, 3 neck ($24/40$), 1L size.
F. – Allihn Condenser, 30cm, $24/40$ joint.
G. – U-Tubes, 20mm tubing, 35/20 ball joint,
   55 ± 5mm centre to centre, 150 ± 5 mm long.
H. – Cross-Over Tube, 35/20 ball joint,
   55 ± 5mm centre to centre, 115 ± mm long.
CHAPTER 4 - FOOD ADDITIVES
SECTION 2: TOTAL PHOSPHORUS

1. SCOPE AND APPLICATION

1.1 The method is applicable to food products in general and can be used to determine added phosphate levels in fish and shellfish which have been treated:

a) with polyphosphate solutions prior to freezing to prevent thaw drip or cracking of glaze in frozen fish and shellfish products;

b) with phosphoric acid as pH adjusting agents in fish protein; and

c) with polyphosphates as sequestering agents in canned seafoods.

2. PRINCIPLE OF THE METHOD

2.1 The samples are dried, followed by mineralizing in a muffle furnace. The ash is dissolved in hydrochloric acid and brought to volume.

2.2 An aliquot is taken and reacted with ammonium molybdo-vanadate forming a stable yellow complex with phosphorus, which is determined colorimetrically at 400 nm.

3. INTERFERENCES

3.1 Incomplete hydrolysis of the sample will result in slow uncontrolled colour development.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.1.1 For frozen fish, comminute the sample and its thaw drip until homogeneous.
5.1.2 For raw fish, fresh or thawed: pass the sample through a grinder a sufficient number of times to obtain a homogeneous blend.

5.1.3 For products that are packed in water, brine or a similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1½ minutes. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

5.2 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from sample, reblend before use.

6. APPARATUS

6.1 Spectrophotometer and matched cuvettes, capable of measuring of absorbance of solutions at 400 nm.

6.2 Muffle furnace.

6.3 Tissue homogenizer, blender, or food grinder.

7. REAGENTS

7.1 Hydrochloric acid (HCl).

7.1.1 Hydrochloric acid solution (1 + 3).

7.2 Nitric acid (HNO₃).

7.3 Ammonium molybdate tetrahydrate ((NH₄)₆Mo₇O₂₄·4H₂O).

7.3.1 Molybdate solution. Dissolve 40 g of ammonium molybdate in 400 mL hot distilled water and cool.

7.4 Perchloric acid (HClO₄, 70%).

7.4.1 Caution: Contact with oxidizable or combustible materials with dehydrating or reducing agents may result in fire or explosion. Persons using perchloric acid should be thoroughly familiar with its hazards.
7.5 Ammonium metavanadate (NH$_4$ VO$_3$).

7.5.1 Metavanadate solution. Dissolve 2 g of ammonium metavanadate in 250 mL hot distilled water. Cool and add 250 mL of 70% HClO$_4$.

7.6 Molybdovanadate reagent. Gradually add molybdate solution to vanadate solution with stirring and dilute to 2 L.

7.7 Potassium orthophosphate monobasic (KH$_2$PO$_4$).

7.7.1 Stock standard solution (2 mg P/ml). Dissolve 8.788 g KH$_2$PO$_4$ and dissolve to 1 L.

7.7.2 Working standard solution (0.1 mg P/ml). Dilute 50 mL of the stock standard solution to 1 L.

8. PROCEDURE

8.1 Accurately weigh 2 g of sample and dry overnight at 100°C. Place sample in a muffle furnace to pre-ash at 250°C, slowly raise the temperature to 350°C and hold at this temperature until all fat is smoked off. Raise the temperature to 600°C for 4 hours.

8.2 Cool, add 40 mL HCl (1 + 3) and several drops HNO$_3$ and bring to a boil.

8.3 Cool, transfer to 200 mL volumetric flask and dilute to volume.

8.4 Filter and place aliquot containing 0.5 mg - 1.5 mg P into a 100 mL volumetric flask. Pipette 0, 5, 8, 10, and 15 mL of the working standard solution into 100 mL volumetric flasks.

8.5 Add 20 mL molybdovanadate reagent, dilute to volume and mix well. Let stand for 10 minutes.

8.6 Set up spectrophotometer as specified by the manufacturer, adjust the wavelength to 400 nm, and read the absorbances of the samples and standard solutions.

9. CALCULATIONS

9.1 Prepare a calibration curve of the absorbance versus the content of the orthophosphate (mg P) in the standard solution.
9.2 Compare the absorbance of the sample solution with the calibration curve (which is plotted versus the total mg P in the 100 mL volumetric flask) to determine the phosphate content (mg P) in the sample aliquot. Calculate percent phosphorus in fish muscle as follows:

\[
\% P = \frac{C}{\text{vol. (mL) of aliquot}} \times \frac{200 \text{ mL}}{\text{weight (g) of fish}} \times \frac{1}{10}
\]

C = phosphorus content (mg P) in the sample aliquot

10 = factor to change mg P/g to % P

9.3 \% P_2O_5 = \% P \times 2.291

2.291 = Conversion factor of P to P_2O_5

9.4 \% Na_2HPO_4 = \% P \times 4.583

4.583 = Conversion factor of P to Na_2HPO_4

10. PRECISION AND ACCURACY

10.1 For a total phosphorus content of 0.4 to 1.0% P_2O_5 the relative standard deviation of 3.9% was found in the 1984 collaborative study. The coefficient of variation for these five samples was 4.0%. The recovery found during the survey was 98.6%.

11. REMARKS

11.1 The natural phosphorus content in fish and shellfish is variable. Therefore, the mean and standard deviation of the natural phosphorus content for a given species needs to be established prior to determining the "total added phosphate".

12. REFERENCES

CHAPTER 4 - FOOD ADDITIVES  
SECTION 3: BORATES

1. SCOPE AND APPLICATION

1.1 The present method may be used to determine the concentration of boric acid and borates added to fish products and by-products, molluscs and crustaceans.

1.2 Boric acid or borates are added to prevent bacterial proliferation in the product.

2. PRINCIPLE OF THE METHOD

2.1 The determination of boric acid and borates added to the product is based on the colour reaction of the principal dye of turmeric (yellow) with boric acid to give a red-brown colour. Any colour change caused by the presence of boric acid and borates should be confirmed by treating the dried red-brown coloured product with alkali to obtain a dark blue-green colour.

3. INTERFERENCES

3.1 In the initial test, several metallic elements, like ferric, molybdic, niobic, tantalic, titanic and zirconium, will yield false positives. These will be eliminated by the alkali confirmation since only the boric complex will yield the dark blue-green colour in alkali.

3.2 Oxidation agents (i.e., peroxides, chromates, nitrates and chlorates) and iodes will interfere with the initial colour development. They can be removed or decomposed by the addition of a reducing agent before the test is conducted.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample and store in such a way so as to maintain its integrity.

5. SAMPLE PREPARATION

5.1 It is important to take into account the type of product and the way it is used and prepared by the consumer.

5.1.1 In the case of products stored in water, brine or a similar
medium that is usually discarded by the consumer, open the package and drain the product over a sieve of an appropriate size for 1 to 1½ minutes. Grind the part of the sample retained by the sieve until a homogeneous mixture is obtained. If the texture of the sample is too hard to be homogenized, pass it through a grinder a number of times to obtain a uniform mixture. The use of a food mill may suffice if the sample is large.

5.1.2 In the case of products that do not contain free liquid, thaw in the package (if the product is frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mixture.

5.1.3 For frozen frosted flesh: defrost and discard the melt water and prepare the sample as described in 5.2.

5.1.4 For frozen products with shell or carapace: remove the head, shell or carapace. Prepare the sample as described in 5.2.

5.1.5 For dried products: prepare the sample as described in 5.2.

5.1.6 For breaded products: remove breading before homogenization.

5.1.7 For fish eggs and caviar, manually stir until homogeneous.

5.2 Grind or mix the sample until homogeneous and place the homogenate in a sealable plastic or glass container. Store in the freezer at -20 °C until use. Ensure that the sample is homogeneous prior to weighing. If a supernatant is present, reblend before use.

6. APPARATUS

6.1 Whatman No. 2 filter paper.

6.2 Whatman No. 41 filter paper.

6.3 Hot plate.

7. REAGENTS

7.1 Purified water: deionized or distilled.

7.2 Ethyl Alcohol Anhydrous (95 % Ethanol), reagent grade.
7.2.1 Ethanol 80%: dilute 84 mL of ethanol with purified water. Dilute to 100 mL.

7.3 Concentrated Hydrochloric Acid (HCl). A.C.S. grade.

7.3.1 HCl 1 N: dilute 8.3 mL of concentrated HCl with purified water. Dilute to 100 mL.

7.4 Concentrated Ammonium Hydroxide (NH₄OH). A.C.S. grade.

7.4.1 NH₄OH 1 N: dilute 6.7 mL of concentrated NH₄OH with purified water. Dilute to 100 mL.

7.5 Turmeric powder (Curcumin), lab grade.

7.6 Turmeric paper: add 100 mL 80% ethanol to 1.5-2.0 g turmeric powder in 250 mL glass-stoppered Erlenmeyer. Shake 5 minutes and filter through a #41 filter paper. Dip sheets of Whatman No. 2 paper into the clear filtrate in flat-bottom dish. Hang paper to dry. After 1 hr cut the paper into 6 x 1 cm strips and store in tightly stoppered container protected from light.

7.7 Boric acid, A.C.S. grade.

7.7.1 Boric acid standard solution: 10 mg H₃BO₃/mL. Dissolve 1.000 g H₃BO₃ in water and dilute to 100 mL.

7.8 Preparation of reference standards: Transfer 0.00, 0.10, 0.20, 0.50, 0.75, 1.00, 2.50, and 5.00 mL standard H₃BO₃ solution to 15 mL test tubes. Dilute to 10 mL with water and add 0.7 mL concentrated HCl. Keep tubes tightly stoppered to prevent evaporation. These standards represent 0.00, 0.02, 0.04, 0.10, 0.15, 0.20, 0.50, and 1.00 % H₃BO₃ in fish (based on 25 g sample extracted with 50 mL water and 10 mL aliquot used for test). Store at 4 °C until use.

8. PROCEDURE

8.1 Weigh 25 g of sample in a 125 mL Erlenmeyer. Add 50 mL of water. Homogenize (with a high-speed homogenizer, food processor or other) or mix until a homogeneous consistency is obtained, free from pieces. Cover the Erlenmeyer with a watch glass or small funnel.

8.2 Bring solution to a boil on hot plate (or over medium
flame) with agitation. Do not overheat.

8.3 Cool in ice bath or in a beaker of water in refrigerator until fat solidifies (ca 0.5 hr). Filter through a plug of glass wool.

8.4 Transfer 10 mL filtrate to 15 mL test tube, add 0.7 mL concentrated HCl, stopper, and mix.

8.5 Mark identification on end of piece of turmeric paper and dip unmarked end into unknown solution to 1/2 the length of paper. Quickly remove moistened paper and place on sheet of white paper. Flat-tipped forceps are useful in handling paper.

8.6 Place freshly prepared standard strips of test paper (made by dipping turmeric papers in a similar manner into a series of standard solutions) alongside sample turmeric strips.

8.7 After >1 hr but <2 hr at room temperature, the strips are dry enough for comparison.

8.8 If Na$_2$B$_4$O$_7$ or H$_3$BO$_3$ is present, the paper turns a characteristic brownish-red.

8.9 Place standards strips ca 1 cm apart on white paper background and colour match the sample strips with the standards strips.

8.10 If colour falls among 2 standards, estimate the value. Disregard the streaks of colour that may develop at edge of test strip.

8.11 If colour intensity is beyond range of standards, repeat test with dilution of filtrate (i.e., 5 mL filtrate, 5 mL water, 0.7 mL HCl and multiply final reading by 2).

8.12 Use a freshly prepared set of standard papers with each series of samples tested.

8.13 Confirmation of reaction:

When the papers have dried, irrespective of the colour reaction, add a drop of 1 N NH$_4$OH. The turmeric papers, if brownish-red, will change to a dark blue-green. Add a drop of 1 N HCl, and if Na$_2$B$_4$O$_7$ or H$_3$BO$_3$ is present the paper will
change to yellowish-red. If negative, the paper will change to yellow.

9. CALCULATIONS

9.1 Compare the colour between the standards and the sample, taking into account the dilution of the samples, if any. Similar colour is related to similar boric acid content.

10. PRECISION AND ACCURACY

10.1 The procedure is semi-quantitative. It will give a reasonable estimation of the boric acid or borates content.

11. REMARKS

11.1 Standard solutions may be stored at 4 °C in test tubes >6 months. On long storage, borate is leached from glassware.

12. REFERENCES


CHAPTER 5 - MISCELLANEOUS
SECTION 1: CANNED TUNA COLOUR MEASUREMENT

1. SCOPE AND APPLICATION

1.1 This procedure is applicable to the measurement of the colour of all canned tuna to determine its colour classification for compliance with Section 49 of the Fish Inspection Regulations P.C. 1971-935 of the Fish Inspection Act (see Appendix A).

2. PRINCIPLE OF THE METHOD

2.1 For routine Inspection purposes, the tuna flesh is compared visually to standardized colour surfaces under a standard light.

2.2 For canned tuna having a borderline colour classification, as determined in 2.1, the tuna flesh colour is determined by using a spectrophotometer to measure its diffuse reflectance at a wavelength of 555 nm.

3. INTERFERENCES

3.1 In 2.1 problems may arise when the flesh colour is considerably different (e.g. pinkish colour) from that of the tiles.

3.2 In 2.2 problems may arise when the flesh is very moist, making it difficult to prepare a smooth diffuse reflecting surface for presentation to the spectrophotometer.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity. Sample must be at room temperature prior to inspection.

5. SAMPLE PREPARATION

5.1 For visual evaluation, open the can and invert on a perforated tray to permit the free aqueous liquor and oil to drain from the flesh.

5.2 If a spectrophotometer measurement is necessary, drain the tuna and place in the cup of the hydraulic press. Place the pressure plate on top of the tuna and position the whole unit in the press. Press the tuna under a pressure of approximately 500 lbs per square inch for 5 minutes.
5.3 Remove the tuna from the cup and shred or grind to a fineness such that it will pass through a 20 mesh sieve.

5.4 Thoroughly mix the tuna after sieving. Place the sieved tuna into the colour measuring cell until two thirds full. Set the glass cover in place and tighten the retaining ring. Screw the pressing plug into the measuring cell (applying pressure to the tuna) until all the air pockets next to the glass cover are removed. Relax the pressure to a point just short of permitting air pockets to reappear, and thoroughly clean the outside of the glass cover to remove all oil and finger prints. The sample is now ready for colour measurement.

6. APPARATUS

6.1 Standard Lighting Unit (for the visual examination of canned tuna), which produces light having a colour temperature of 6470°K and equivalent to C.I.E. Illuminant C.

6.2 Tuna colour standards, one equivalent to the colour boundary between white and light tuna (a diffuse reflectance of 33.7% of that of MgO at 555 nm), and a second equivalent to the colour boundary between light and dark tuna (a diffuse reflectance of 22.6% that of MgO at 555 nm). Coloured porcelain-on-steel tiles similar to the tiles discussed in reference 12.2 have proved to be satisfactory.

6.3 Support for the colour standards to permit the comparative viewing of the samples and standards under the standard light. Mount the standards at an angle that will permit the inspector to view them without encountering light glare.

6.4 Hydraulic press for squeezing the excess moisture from the tuna flesh. The press must be equipped with various sized cups to hold the different sized tuna packs.

6.5 No. 20 sieve.

6.6 Colorimeter or spectrophotometer equipped with a diffuse reflectance attachment and capable of measuring the colour of solids at 555 nm.

6.7 Colour measuring cell or sample holder. Details of a suitable design for use with the B & L 505 spectrophotometer is shown on the attached figure.
7. **REAGENTS**

7.1 Magnesium ribbon to prepare the standard white reflectance surfaces necessary to calibrate the spectrophotometer for 100% reflectance. Any material that will give a diffuse reflectance of at least 99% that of MgO at 555 nm may be used as an alternative reflectance standard.

8. **PROCEDURE**

8.1 Remove the drained tuna sample from the can, and break across the tuna loins or flakes so that a fresh surface is available for colour viewing. View the colour of the flesh under the standard light and compare visually to the two colour standards. Tuna that is equal in colour to, or lighter than the White-Light Tuna Standard is classified as white meat tuna. Tuna which is equal to, or lighter than the Light-Dark Tuna Standard, but is darker than the White-Light Standard is classified as light meat tuna. All other tuna is dark meat tuna.

8.1.1 Classification of cans containing more than one piece of fish: If 15 to 20% or more of the flesh is obviously darker than the declared colour when compared to the standard colour boundary, the sample is considered as darker than the standard. If less than 15% of the flesh in the can is darker than the declared colour, the sample is classified according to the average colour of the flesh.

8.1.2 If 10% or more of the samples are rated below the colour designations on the label, the lot is reclassified to the lower colour designation.

8.1.3 If the colour classification of the lot is in doubt after examination by two inspectors, then all the cans of questionable colour are submitted to colour measurement by the spectrophotometer.

8.2 Spectrophotometric measurement: The directions that follow are for a Bausch & Lomb 505 Recording Spectrophotometer. If an equivalent instrument is used, the directions will have to be modified to make them compatible to that instrument.

8.2.1 Preparation of white reflectance standard: Prepare two standards by burning magnesium ribbon and holding a plane optical glass lens (identical to the one used in the sample holder described in 6.8) in the smoke above the flame. The side of the lens to be coated with MgO is the same side that would be in contact with the tuna flesh if it were used in the sample holder. Coat MgO on the lens until a completely opaque layer is obtained (estimated at approximately 1 to 2 mm). Do not hold
the lens too close to the flame or the condensed smoke will not be absolutely white. Position the two coated lenses in the spectrophotometer over the standard and the sample viewing ports (uncoated side toward the viewing port). Ensure that the MgO file is not touched.

CAUTION: Safety goggles are required for protection from the UV light when burning magnesium ribbon.

8.2.2 Set up the spectrophotometer according to the manufacturer's instructions and adjust the gain to give 100% reflectance at 555 nm. Record the spectral curve over the range of visible wavelengths to check that the instrument is adjusted to give 100% reflectance at all wavelengths. Interchange the two standard whites to the opposite viewing ports and rerun the 100% reflectance curve. The two recorded curves should differ by less than 1% over the entire visible wavelength range. If such is not the case, recoat the standard white having the lowest reflectance with another thin layer of MgO and run two more spectrophotometric curves as described above. Repeat the coating of one or both of the standard whites until the recording of both curves vary by less than 1%. The standards may be useable for a period of up to one week depending upon storage and atmospheric conditions and the density of the prepared surface. Check daily to see that their variance is less than 1% over the entire visible wavelength range.

8.2.3 Remove the white standard from the sample-viewing port, position in its place the tuna sample as prepared in 5.4 and record the diffuse reflectance curve of the sample.

9. **CALCULATIONS**

9.1 Read the percent reflectance at 555 nm from the recorded spectrophotometric curve.

9.2 The borderline between white and light meat tuna is 33.7% of the diffuse reflectance of MgO and the borderline between light and dark meat tuna is 22.6% of the reflectance of MgO at 555 nm. However, to allow for colour changes in the standards, and for instrument error, a tolerance of 2% reflectance is permitted.
10. **PRECISION AND ACCURACY**

10.1 In the visual examination of canned tuna, it has been found that all samples having a colour difference of more than ± 2% luminous reflectance from a colour boundary would be classified correctly by an inspector.

10.2 Spectrophotometric Method.

10.2.1 If the reflectance curve of a sample is remeasured after rotating the sample holder 90°, the second recording will not differ from the first trace by more than the width of the recorder pen line.

10.2.2 If the ground, screened and mixed sample is packed into two different sample holders, the spectrophotometric traces from these two prepared samples will normally differ by less than 1/2 of 1% at 555 nm.

11. **REMARKS**

11.1 The preparation of the standard white reference colour is critical and time consuming if magnesium oxide (MgO) is used. If another standard material is used, the colour measurement of the sample must be corrected to take into account the difference in colour between the standard used and MgO.

12. **REFERENCES**

12.1 Fish Inspection Regulations of the Fish Inspection Act, P.C. 1971-935.

APPENDIX A

Section 49, Fish Inspection Regulations

49. In addition to the requirements of section 25, the labels on all cans of tuna shall indicate the colour of the fish flesh in accordance with the following colour classifications:

(a) "White Meat Tuna" or "White Tuna" is canned tuna that has diffuse luminous reflectance of not less than thirty-three point seven per cent that of magnesium oxide when that reflectance is measured by a method prescribed by the Minister;

(b) "Light Meat Tuna" or "Light Tuna" is canned tuna that has a diffuse luminous reflectance of not less than twenty-two point six per cent that of magnesium oxide when that reflectance is measured by a method prescribed by the Minister; and

(c) "Dark Meat Tuna" or "Dark Tuna" is canned tuna that does not meet the colour requirements of "Light Meat Tuna".
FISH SAMPLE HOLDER

OPTICAL GLASS

1/16 X 1 3/4 I.D.
RUBBER O RING

32 THREADS/IN.

BRASS RING

20 THREADS/IN.
LOOSE FIT

BRASS PLUG

1/4" DIA. HOLE
IN CENTER

PLASTIC PLUG

1/16" DIA. DRAIN HOLE
IN ONE SIDE

SCALE: DOUBLE ACTUAL SIZE
CHAPTER 5 - MISCELLANEOUS

SECTION 2: PARALYTIC SHELLFISH POISON EXTRACTION PROCEDURE

1. SCOPE AND APPLICATION
1.1 The procedure is applicable to molluscan shellfish in the live, frozen or processed state.

2. PRINCIPLE OF THE METHOD
2.1 Shellfish do not produce the paralytic poison; they acquire it by feeding on certain species of marine dinoflagellates.
2.2 The amount of toxin present is determined by preparing dilute acid extracts of the meats, injecting them into mice, establishing the time it takes injected mice to die, and calculating the toxin level on the basis of prepared known standards.

3. INTERFERENCES
3.1 High concentrations of salt such as is found in salted molluscan shellfish will react on the nerve impulses of mice.

4. SAMPLING PROCEDURE AND STORAGE
4.1 Live samples.
4.1.1 Collect a minimum of 6 animals or enough to yield 200 g of meat.
4.1.2 Pack sample in a shipping container with adequate protection against mechanical damage and temperature change. Do not pack with ice in direct contact with shellfish.
4.1.3 Place in the package a description of the sample, i.e., date of collection, time, location, species, and any other pertinent data.
4.1.4 Send to the laboratory without delay. Samples from remote areas may be frozen.
4.2 Canned, frozen and breaded shellfish.
4.2.1  Take a representative sample from the product lot and store so as to maintain sample integrity.

5.  SAMPLE PREPARATION

5.1  Live samples.
5.1.1 Thoroughly clean outside of shellfish with fresh water.
5.1.2 Open by cutting adductor muscles.
5.1.3 Thoroughly rinse meat with fresh water to remove foreign material.
5.1.4 Remove meat from shell without cutting or damaging body.
5.1.5 Collect ca 150 g of meat.
5.1.6 Transfer meats to a No. 10 mesh sieve without layering and drain for five minutes; discard drainings.
5.1.7 Mascerate meats in tissue homogenizer until a homogeneous slurry is obtained.

5.2  Canned shellfish.
5.2.1 Blend entire contents of can as in 5.1.7.

5.3  Frozen shellfish.
5.3.1 If frozen in shell, thaw and treat as in 5.1.1 to 5.1.7.
5.3.2 If frozen shucked, thaw and treat as in 5.1.5 to 5.1.7.
5.3.3 If frozen shucked and drained, thaw and treat as in 5.1.7.

5.4  Breaded shellfish.
5.4.1 Remove breading and treat as in 5.1.7.

6.  APPARATUS

6.1  Sieve, No. 10 mesh.
6.2  Centrifuge.
6.3 pH meter.

7. REAGENTS

7.1 Hydrochloric acid (HCl).

7.1.1 HCl solution (0.18N). Dilute 15 mL conc HCl to 1L with distilled water.

7.1.2 HCl solution (6N). Dilute conc HCl 1 + 1 with distilled water.

7.2 Sodium hydroxide (NaOH).

7.2.1 NaOH solution (0.1N). Dissolve 4 g NaOH in distilled water and dilute to 1L. (A.O.A.C,12ed, (1975), procedure 50.034.)

8. PROCEDURE

8.1 Accurately weigh a 100 g of sample homogenate in a 400 mL tared beaker graduated at the 200 mL level.

8.2 Add 100 mL 0.18N HCl and stir thoroughly. Check that the pH is 3.5 ± 0.5, and adjust if necessary.

8.3 Heat mixture to boiling as quickly as possible and with constant stirring (ca 5 - 7 min), boil gently five minutes and let cool to room temperature.

8.4 Cool the digest, add distilled water to return weight to 200 g and adjust the pH to 3.3 - 3.8 (using 6N HCl or 0.1N NaOH as necessary) with constant stirring.

8.5 Stir mixture to homogeneity and allow to settle until portion of supernatent is translucent and can be decanted free of solid particles large enough to block a 26-gauge hypodermic needle. Alternatively, centrifuge mixture or supernatent five minutes at 3000 rpm, or filter. Only enough liquid to perform bioassay is required.

8.6 Decant ca 15 mL of the supernatent liquid into a clean sample bottle, close securely with a waxed cork, affix identification label and number, pack securely in a suitable mailing container, and send to:
9. CALCULATIONS

9.1 Health Protection Branch will do all calculations involved and report results as micrograms PSP per 100 g of shellfish meat unless otherwise advised by sender.

10. PRECISION AND ACCURACY

10.1 Accuracy of a three mouse test is considered to be ±20%, while that of a six mouse test is ca ± 10%.

11. REMARKS

11.1 On the Canadian Atlantic coast, the problem is confined to the middle and lower reaches of the Bay of Fundy and the lower estuary to the St. Lawrence River.

11.2 On the Canadian Pacific coast the areas affected are not predictable. However, outbreaks generally occur in sheltered areas.

11.3 Areas affected by PSP usually show great seasonal and annual variations in shellfish toxicity. Typically, inshore species like soft shell clams and mussels are poison-free, or nearly so, during winter and spring but their toxicity can rise quickly to a peak in summer and then decline in autumn. (There is little consistency in annual variation). When Pacific Coast butter clams become toxic, they may take years to purge themselves of the toxin.

12. REFERENCES


CHAPTER 5 - MISCELLANEOUS
SECTION 3: SPECIES IDENTIFICATION BY ELECTROPHORESIS

1. SCOPE AND APPLICATION

1.1 This method is applicable to a large variety of fish species and is based on a visual comparison of the characteristic patterns formed when the water soluble protein extracts of known and unknown fish species are electrophoresed.

2. PRINCIPLE OF THE METHOD

2.1 An extract of water soluble proteins is applied to a cellulose poly-acetate medium to which a direct current is applied. The components of the extract migrate through the pore structure of the supporting medium which acts as a molecular sieve such that proteins of the same charge to mass ratio separate if their molecular sizes are sufficiently different.

3. INTERFERENCES

3.1 There are no known interferences.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

4.2 Samples can be fresh, frozen, smoked or salted; but heat processed samples are unsuitable.

4.3 Samples may be held for several months in frozen storage.

4.4 At no time during the analysis should the temperature of a sample or sample extract be allowed to increase to the point where protein will denature.

5. SAMPLE PREPARATION

5.1 Fresh: Freeze the sample and use the undiluted drip from a thawed portion.
5.2 Frozen: Thaw the sample and use the undiluted drip.
5.3 Freeze-dried: Reconstitute sample with water and treat as a fresh sample.
5.4 Smoked and salted fish products require special treatment (see REMARKS).

6. APPARATUS
6.1 Electrophoresis cabinet.
6.2 Serum applicator.
6.3 Cellulose polyacetate strips, 2.5 cm x 15 cm.
6.4 Absorbent paper, 20 x 30 cm.
6.5 Magna-Grip Tensioners.
6.6 Micro Pipets.
6.7 Staining Tray.
6.8 Strip Soaking Tray.
6.9 D.C. Power Supply, 0-500V, 0-25 mA.

7. REAGENTS
7.1 2-Amino-2-hydroxymethyl-1,3-propanediol (TRIS).
7.2 Ethylenediaminetetraacetic acid (EDTA).
7.3 Boric acid.
7.4 Diethylbarbituric acid.
7.5 Sodium diethylbarbiturate.
7.6 Methanol.
7.7 Acetic acid, glacial.
7.8 Amino black 10B.
7.9 Trichloroacetic acid (TCA).

7.9.1 TCA solution (5%). Used as washing solution.

7.10 Ethyl alcohol (95%).

7.11 Buffer solution. Any of the following three buffer solutions can be used:

7.11.1 Dissolve 60.5 g TRIS, 6.0 g EDTA and 4.6 g boric acid in distilled water and dilute to 1L. Dilute 250 mL of this stock solution to 1L to use as a working solution.

7.11.2 A.O.A.C. buffer solution. Dissolve 8.64 g sodium diethylbarbiturate and 1.2 g diethylbarbituric acid in distilled water and dilute to 1L. (The buffer is pH 8.6 with an ionic strength of 0.04M.)

7.11.3 H.R. Buffer (Gelman, product no. 51104). Dissolve one vial (18 g) in 1800 mL distilled water.

7.12 Staining solution. Either of the following two staining solutions can be used:

7.12.1 Mix 1000 mL methanol, 1000 mL distilled water and 200 mL glacial acetic acid, and add 25 g amino black 10B.

7.12.2 Ponceau S Stain (Gelman). Dissolve one capsule (20 mg) Ponceau S Stain in 100 mL 5% TCA solution.

7.13 Clearing solution. Mix 15 mL glacial acetic acid with 85 mL 95% ethyl alcohol.

8. PROCEDURE

8.1.1 Soak cellulose polyacetate strips a minimum of 30 minutes in buffer solution.

8.1.2 Add chilled buffer (1°C) to each chamber of cabinet and level to point slightly below compartment dividers.

8.1.3 Remove a strip from the buffer and gently blot between sheets of absorbent paper to remove excess buffer.
8.1.4 Fill the micro-pipet with the sample extract and transfer to the applicator. Leave 3 mm on each size of applicator free of sample.

8.1.5 Press the applicator firmly against the strip about 5 cm from one end.

8.1.6 Place the strip containing sample across cabinet dividers so that the sample is on cathode side and both ends are immersed in buffer in the two outer chambers.

8.1.7 Secure the strip at each end with magna-grips and keep taut. Repeat steps 8.1.3 to 8.1.7 for each strip.

8.1.8 Set up the apparatus according to the manufacturer's instructions. Put cabinet cover in place, connect electrodes and set D.C. power supply at a potential of at least 300V, and a current of at least 1 mA per strip. Apply the current for 45 minutes.

8.1.9 Shut off the power supply, remove the strip from cabinet, and place in the staining solution 2-5 minutes.

8.1.10 Immerse the strip in a series of three 5% acetic acid rinse solutions to remove excess dye. Rinse the strip until only the protein bands are left stained and remainder of the strip is free from dye. (A strip may be left in 5% acetic acid solution for 2-3 weeks.)

8.1.11 Compare the protein pattern of the sample with those of known species of fish. Standard should be run simultaneously.

8.2 If a permanent slide of the protein pattern is desired:

8.2.1 Place the strip on a 2.5 x 11.5 cm glass slide and dry in air.

8.2.2 Dip the glass slide and strip into the clearing solution for 20-30 seconds. Remove entrapped air bubbles.

8.2.3 Allow the slide to dry. If the strip does not completely clear, repeat steps 8.2.1 and 8.2.2.

9. **CALCULATIONS**

9.1 Visually compare the protein pattern of the unknown sample with patterns from known species of fish.
10. PRECISION AND ACCURACY

10.1 With marine species, this procedure has proven to be reliable.

11. REMARKS

11.1 To help control heat build-up and to reduce evaporation, buffer solution should be kept refrigerated until ready for use. After an electrophoresis run, the buffer solution in the cabinet should be poured back into the stock bottle and refrigerated. Buffer should be changed every ten runs or at least once a week.

11.2 Temperature should be controlled such that there is no condensation build-up in the electrophoresis cabinet.

11.3 When performing species identification on smoked and salted fish products using electrophoretic techniques, greater resolution, and thus a much improved electrophoretic pattern, can be obtained by doing the following:

(i) homogenize the sample with a 10 mM equivalent of dithiothreitol;

(ii) centrifuge for 5-10 minutes at 3000 rpm; and

(iii) subject the resultant supernatant to electrophoresis.

Because of its low redox potential (-0.33 volts at pH 7), dithiothreitol is capable of maintaining monothiols completely in the reduced state and reducing disulfides quantitatively (see reference 12.2).

12. REFERENCES


CHAPTER 5 - MISCELLANEOUS
SECTION 4: DOMOIC ACID EXTRACTION AND ANALYSIS PROCEDURE

1. SCOPE AND APPLICATION

1.1 This procedure is applicable to molluscan shellfish, crustaceans and finfish in the live, frozen or processed state. The method has been used successfully to determine domoic acid (DA) concentrations in shellfish tissues ranging from 0.5 µg/g to over 4000 µg/g.

2. PRINCIPLE OF THE METHOD

2.1 Shellfish do not produce the toxin; they accumulate it by feeding on certain species of marine dinoflagellates. Other fish such as lobsters, crab and finfish acquire the toxins by feeding on prey that are contaminated with the toxins.

2.2 The amount of toxin present is determined by preparing dilute acid extracts of the meats, subjecting the extract to HPLC analysis and calculating the toxin level on the basis of prepared standards of known concentration.

3. INTERFERENCES

3.1 The amino acid Tryptophan has been reported to cause some interference with the analysis of domoic acid in other procedures. To date there has not been any evidence to indicate any interferences in this procedure.

3.2 The analysis of fatty fish such as mackerel and herring may eventually have detrimental affects on the performance of the HPLC column which can be alleviated by flushing with methanol.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Live samples

4.1.1 Collect a minimum of 5 animals (10-12 animals for molluscan shellfish) or enough to yield 150 g of meat.

4.1.2 Pack sample in a shipping container with adequate protection against mechanical damage and temperature change. Do not pack with ice in direct contact with shellfish.
4.1.3 Place in the package a description of the sample, i.e., date of collection, time, location, species, and any other pertinent data.

4.1.4 Send to the laboratory without delay.

4.2 Canned, frozen and breaded shellfish, finfish and crustaceans

4.2.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLE PREPARATION

5.1 Live samples

5.1.1 Thoroughly clean outside of shellfish with fresh water.

5.1.2 Open by cutting adductor muscles.

5.1.3 Rinse meat with fresh water to remove foreign material.

5.1.4 Remove meat from shell without cutting or damaging body.

5.1.5 Collect ca 150 g of meat.

5.1.6 Transfer meats to a No. 10 mesh sieve without layering and drain for five minutes; discard drainings.

5.1.7 Macerate meats in tissue homogenizer until a homogeneous slurry is obtained.

5.2 Canned shellfish

5.2.1 Blend entire contents of can as in 5.1.7.

5.3 Frozen shellfish

5.3.1 If frozen in shell, thaw and treat as in 5.1.1 to 5.1.7.

5.3.2 If frozen shucked, thaw and treat as in 5.1.5 to 5.1.7.

5.3.3 If frozen shucked and drained, thaw and treat as in 5.1.7.

5.4 Breaded shellfish

5.4.1 Remove breading and treat as in 5.1.7.
5.5 **Finfish, Crustaceans**

5.5.1 Commute the sample until homogeneous.

6. **APPARATUS**

6.1 Sieve, No. 10 mesh.

6.2 Centrifuge.

6.3 pH meter.

6.4 Polytron homogenizer or equivalent.

6.4.1 Vortex mixer.

6.5 3 mL disposable syringes (B-D).

6.6 25 mm nylon syringe filters (0.2 µ, recommended).

6.7 Pipettors.

6.7.1 Pipettors with 1.0, 0.75, and 0.5 mL capacities.

6.8 Isocratic HPLC Pump capable of providing reliable flows up to 1.5 mL/min.

6.9 Autosampling system able to communicate with pump and data system and provide up to 20 µL injection volumes.

6.10 Vydac 201 TP54 reverse phase HPLC column 25 cm x 4.6 mm I.D.

6.11 Column oven capable of maintaining the column temperature at 50 °C.

6.12 HPLC UV monitor, wavelength 242 nm.

6.13 Data handling system and recording device.

7. **REAGENTS**

7.1 Hydrochloric acid (HCl).

7.1.1 HCl (0.1N) solution. Dilute 8.3 mL conc. HCl to 1L with distilled water.
7.1.2 HCl (5N) solution. Dilute 1 part conc. HCl with 1.4 parts distilled water.

7.2 Sodium hydroxide (NaOH).

7.2.1 NaOH (0.1N) solution. Dissolve 4 g NaOH in distilled water and dilute to 1L (A.O.A.C., 12th ed., (1975), procedure 50.034.)

7.3 Methanol, ACS grade.

7.4 Trifluoroacetic acid (TFA), ACS grade.

7.5 Acetonitrile, HPLC grade.

7.6 Glass-distilled water.

7.7 Mobile Phase.

7.7.1 Mobile Phase (Routine) - 11% Acetonitrile + 0.1% TFA in glass-distilled water.

7.7.2 Mobile Phase (Confirmatory) - 8% Acetonitrile + 0.1% TFA in glass-distilled water.

7.8 DACS - Certified calibration standard, NRC, Halifax.

7.9 Stock Standard (A) - Approximately 50 mg DA (Diagnostic Chemicals Ltd.) dissolved in 50 mL 10% methanol (1.0 mg DA/mL). The standard is calibrated against the DACS standard to determine the precise concentration. This less expensive standard is used to prepare working standards for day-to-day use as well as for preparing spiked samples.

7.9.1 Dilute Stock Standard (B), (100 µg DA/mL 25% methanol); 5 mL DA stock standard (A) diluted to 50 mL with 25% methanol.

7.9.2 Working Standards

7.9.2.1 High Standard (6.0 µg DA/mL); 6.0 mL dilute stock standard (B) diluted to 100 mL with 25% methanol.

7.9.2.2 Middle Standard (1.0 µg DA/mL); 1.0 mL dilute stock standard (B) diluted to 100 mL with 25% methanol.
7.9.2.3 Low Standard (0.06 µg DA/mL);
1.0 mL high standard diluted to 100 mL with 25% methanol.

A single point calibration has also been used as the linear regressions over the proposed range of concentrations is quite linear.

8. PROCEDURE

8.1 Domoic Acid Extraction Procedure (Screening Analysis)
This is based on the Association of Official Analytical Chemists (AOAC) method "Paralytic Shellfish Poison Biological Method Final Action" (AOAC, 16th Ed., 35.1.37).

8.1.1 Accurately weigh 100 g of sample homogenate in a 400 mL tarred beaker graduated at the 200 mL level.

8.1.2 Add 100 mL 0.1N HCl and stir thoroughly. Check that the pH is 3.5 ± 0.5, and adjust if necessary.

8.1.3 Heat mixture to boiling as quickly as possible (ca 5 - 7 min.), cover with a watch glass or equivalent and boil at 100 °C with constant stirring. Boil for five minutes and let cool to room temperature.

8.1.4 Cool the digest, adjust the pH to 3.3 - 3.8 (using 5N HCl or 0.1N NaOH as necessary) with constant stirring and add distilled water to return weight to 200 g.

8.1.5 Stir mixture until homogeneous and allow to settle until a portion of the supernatant is translucent and can be decanted free of solid particles large enough to block a 26-gauge hypodermic needle. Alternatively, centrifuge mixture or supernatant five minutes at 3000 rpm, or filter.

8.1.6 Decant ca 15 mL of the supernatant liquid into a clean scintillation vial, close securely, affix identification label and number. The extract is suitable for both the PSP Bioassay and domoic acid analysis. Samples can be stored in a refrigerator (at 4 °C) until the analysis.

8.1.7 Remove samples from the refrigerator and allow to come to room temperature.

8.1.8 Pipette 1.0 mL of each sample into a 13 x 100 mm culture tube containing 1.0 mL of ACS grade methanol and mix.
8.1.9 Centrifuge at 1200 RPM for 10 minutes.

8.1.10 Draw approximately 1.5 mL into a 3 mL disposable syringe and filter through a syringe filter into a 13 x 100 mm culture tube.

8.1.11 Pipette 0.75 mL of each filtered sample into separate autosampler vial and dilute with 0.75 mL of glass-distilled water, cap and mix. Proceed to section 8.3.

8.2 Domoic Acid (Critical Analysis) Methanol Extraction Procedure

This procedure should be utilized on samples that are suspected to contain significant levels of domoic acid or on samples which do not require PSP analysis.

8.2.1 Weigh 5.0 g of sample homogenate in a 50 mL screw cap culture tube.

8.2.2 Add 5.0 mL of distilled water and mix with a vortex mixer.

8.2.3 Add 10.0 mL Methanol (ACS) and mix with the vortex mixer.

8.2.4 Centrifuge the sample at 1000 RPM for 10 minutes and decant the supernatant into a scintillation vial.

8.2.5 Draw approximately 1.5 mL into a 3 mL disposable syringe and filter through a syringe filter into a 13 x 100 mm culture tube.

8.2.6 Pipette 0.75 mL of each filtered sample into separate autosampler vial and dilute with 0.75 mL of glass-distilled water, cap and mix. Proceed to section 8.3.

8.3 HPLC Analysis

8.3.1 Equilibrate HPLC system at a column oven temperature of 50 °C with fresh mobile phase for 20 minutes. Adjust the mobile phase or column temperature as required to obtain retention times of the standards between 9 and 11 minutes.

HPLC Conditions: Mobile Phase - 11 % Acetonitrile + 0.1% TFA in glass-distilled water

    Flow - 1.0 mL/min.
    Wavelength - 242 nm
    Run Time - 12-15 min.
8.3.2 Calibrate the instrument by repeated injections of the working standards.

8.3.3 Inject samples, blanks and certified reference materials and measure the resulting peak heights obtained from the integrator.

9. CALCULATIONS

9.1 Measure peak heights of the standards and prepare a calibration curve of the amount of domoic acid per injection versus peak heights.

9.2 From the linear regression of the curve determine the amount (ng) of domoic acid present in each injection of sample.

9.3 Calculate the level of domoic acid in the sample using the following formula:

\[
\mu g/g = \frac{ng/injection \times D}{\mu L \text{ injected}}
\]

D = Dilution factor (the normal dilution factor for this procedure is 8).

10. PRECISION AND ACCURACY

10.1 Data indicates that the reproducibility is approximately ± 10% at the 20 µg/g level for the methanol extract and ± 13% for the PSP type extract.

10.2 The detection limit is approximately 0.5 µg/g.

11. REMARKS

11.1 Representative chromatograms of a domoic acid standard and domoic acid extracted from clam homogenate are shown in Appendix A, Figures 1 and 2, respectively. A calibration curve of the working standards is shown in Figure 3 while the chromatogram in Figure 4 shows the baseline separation of domoic acid and tryptophan standards.

11.2 The methanol extract percentage recovery of domoic acid can be expected to be 95% or better for clam, mussel, oyster and scallop homogenates.
11.3 The routine PSP type extract gives comparable recoveries for scallop and oyster homogenates but only 80% of the methanol extract values for mussels and 45% of the methanol extract values for clams.

11.4 The PSP type extract, while convenient as a screening tool for samples which must also be analyzed for PSP should not be used for domoic acid analysis when analytical accuracy is necessary.

11.5 Recommended storage is in a refrigerator at 4 °C. Acidic extracts should not be frozen as the increased concentration of acid during freezing may destroy some of the domoic acid. Extracted domoic acid is more stable in the water-methanol extract, however, both extracts appear to offer adequate stability during a one-week storage period at room temperature.

11.6 Methanol is used after the PSP type extraction to precipitate much of the water soluble protein. This additional cleanup step removes material that would otherwise affect column performance and longevity.

11.7 Samples containing significant DA or samples from areas or species showing DA for the first time should be confirmed either chromatographically or by using a diode array detection. Conditions for diode array confirmations are outlined in Appendix B.

12. REFERENCES


Figure 1. HPLC UV trace (242 nm) of a domoic acid standard (21.2 ng) run on a Vydac 201 TP54 (25 cm x 4.6 mm I.D.), 5µ, RP-18 column. Operating conditions, Oven temp. 50 °C, flow rate 1.0 mL/min. Isocratic - 11% acetonitrile in glass-distilled water with 0.1% trifluoroacetic acid added as a modifier.
Figure 2. HPLC UV trace (242 nm) of a methanol extract of clam tissue (7.3 µg DA/g). Conditions as in Figure 1.
DOMOIC CALIBRATION CURVE

Figure 3. Calibration curve for domoic acid run on the Vydac column. Conditions as in Figure 1.
Figure 4. HPLC UV trace (242 nm) of standard DA (4.7 ng) and standard tryptophan (14.6 ng). Conditions as in Figure 1.
APPENDIX B

Diode Array Confirmation

Conditions for the HPLC analysis are the same as those outlined in section 8.3 with the exception of diode array detection (DAD). The signal is monitored at 242 nm with a bandwidth of 40. Spectra are stored from 190 to 600 nm at 2 nm increments from seven minutes into the run to end run. Spectra obtained from standard DA are compared with those obtained from sample runs.

The spectra obtained from a sample of soft-shelled clams containing 6 µg DA/g is shown in Figure 1.

Figure 1. HPLC DAD spectra of a soft-shelled clam sample containing 6 µg DA/g. HPLC operating conditions as in Appendix A, Figure 1. DAD spectra collected from 190 to 600 nm at 2 nm intervals.
CHAPTER 5 - MISCELLANEOUS

SECTION 5: DIARRHETIC SHELLFISH TOXINS (DSP) EXTRACTION AND ANALYSIS PROCEDURE

1. SCOPE AND APPLICATION

1.1 This procedure is applicable to molluscan shellfish in the live, frozen or processed state. The method has been successfully used to determine okadaic acid (OA) and dinophysis toxin-1 (DTX-1) in shellfish tissues. The method has a detection limit of 0.15 ng for each analyte which under the conditions described is equivalent to 0.03 µg/g in the tissue.

2. PRINCIPLE OF THE METHOD

2.1 While shellfish do not produce toxins naturally, they are able to accumulate sufficiently high levels to cause illness in the consumer by feeding on certain species of marine dinoflagellates.

2.2 The DSP toxins are extracted into methanol from the digestive glands of the shellfish. Non-polar lipids are removed from the extract with hexane, the toxins partitioned into chloroform and the extract taken to dryness under nitrogen. A portion of the residue is reacted with commercial 9-anthryldiazomethane (ADAM) reagent to form a fluorescent derivative. The derivative is cleaned up on a silica cartridge column and analyzed by HPLC with fluorescence detection. The toxin levels are calculated on the basis of prepared standards of known concentrations.

3. INTERFERENCES

3.1 There are a number of compounds that may cause interference with the analysis. For example, compounds such as free fatty acids can produce fluorescent derivatives when reacted with ADAM reagent. Such compounds may be present in the organic extract. These interferences seem to be seasonal in nature and appear for the most part during the late summer and early fall. Strict attention to the quality of the chromatography and quality control in general is necessary to ensure proper quantitation of the analytes. A method used for the cleanup of "dirty" samples can be found in Appendix B.
4. **SAMPLING PROCEDURE AND STORAGE**

4.1 Live samples.

4.1.1 Collect a minimum of 10-12 animals or enough to provide at least 20 g of digestive gland.

4.1.2 Pack the samples in a shipping container with adequate protection against mechanical damage and temperature changes. Do not pack with ice in direct contact with shellfish.

4.1.3 Place a description of the sample in the package, i.e., date of collection, time, location, species and any other pertinent data.

4.1.4 Send to the laboratory without unnecessary delay.

4.2 Canned or frozen shellfish samples.

4.1.5 Take a representative sample of the lot and store so as to maintain sample integrity.

5. **SAMPLE PREPARATION**

5.1 Live samples.

5.1.1 Thoroughly clean outside of shellfish with fresh water.

5.1.2 Open by cutting adductor muscle.

5.1.3 Rinse meat with fresh water to remove foreign material.

5.1.4 Remove meat from the shell without cutting or damaging body.

5.1.5 Dissect out the digestive gland.

5.1.6 Collect ca 20 g of digestive glands.

5.1.7 Homogenize the digestive glands in a tissue homogenizer until a uniform slurry is obtained.

5.2 Frozen shellfish.

5.2.1 Thaw and treat as in 5.1.1 to 5.1.7.
5.3  Canned shellfish.
5.3.1 Treat contents of can as in 5.1.5 to 5.1.7.

6. **APPARATUS**

6.1 Polytron homogenizer or equivalent.
6.2 Pipettors.
6.2.1 100-1000 µL variable volume pipettor.
6.2.2 10-100 µL variable volume pipettor.
6.2.3 1-5 mL variable volume pipettor.
6.3 Centrifuge.
6.4 Nitrogen evaporator or equivalent.
6.5 Vortex mixer.
6.6 Fluorimeter: variable wavelength (excitation approx. 350 nm; emission approx. 440 nm).
6.7 Vacuum manifold (optional).
6.8 Silica cartridges containing 690 mg silica, pH 7, pore size 125 Å, 80 µm particle size.
6.9 HPLC pump system capable of generating reliable, rapid binary gradients and flows up to 1.1 mL/min.
6.10 Autosampling system able to communicate with the pump and data system and provide up to 10 µL injections.
6.11 Column oven capable of maintaining the column temperature at 35 °C.
6.12 HPLC fluorescence monitor with an excitation wavelength of 249 nm and an emission wavelength of 407 nm.
6.13 Data-handling system and recording device.
6.14 HPLC columns:
6.14.1 Routine HPLC column: Superspher 100 RP-18, 3 µ, 12.5 cm x
2 mm i.d., used without a guard column.

6.14.2 Confirmation HPLC column: Lichrospher 100 RP-18, 5 µ, 25 cm x 4 mm i.d., used without a guard column.

7. **REAGENTS**

7.1 Glass distilled water (GDW).
7.2 Acetonitrile, HPLC grade.
7.3 Methanol, HPLC grade.
7.4 Chloroform (CHCl₃), ACS grade.
7.5 Hexane, pesticide grade.
7.6 Ethanol (95 %), ACS grade.
7.7 OACS - Certified reference standard, NRC, Halifax.
7.8 MUS2 - Certified reference material, NRC, Halifax.
7.9 Okadaic acid (OA): Diagnostic Chemicals Ltd..
  7.9.1 Stock standard (0.1 µg/µL):
    25 µg of OA standard diluted to 250 µL with methanol.
  7.9.2 Working standard (5 ng OA/µL):
    10 µL of stock standard diluted to 200 µL with methanol.
7.10 Hyodeoxycholic acid (HDC), ACS grade.
  7.10.1 Stock standard (0.1 mg HDC/mL):
    10 mg HDC dissolved in 95% ethanol diluted to 100 mL.
  7.10.2 Working standard (5 ng HDC/µL):
    0.5 mL of stock standard diluted to 10 mL with 95 % ethanol.
7.11  *Prorocentrum lima* extract stock solution (see 11.4, 3500 µg OA/mL, 520 µg DTX-1/mL):

The solution is a concentrated extract from laboratory cultured *Prorocentrum lima*. The solution (in 95 % Ethanol) contains both OA and DTX-1 and is standardized for OA against the certified reference standard OACS. DTX-1, for the purposes of quantitation is considered to generate an equivalent response to OA.

7.11.1 *Prorocentrum lima* extract working solution (7.61 µg OA/mL): 10 µL of *Prorocentrum lima* stock solution diluted to 4.6 mL with methanol.

7.12  9-Anthryldiazomethane (ADAM), Molecular Probes, Eugene, Oregon.

7.12.1 ADAM stock:

1 mg portions of ADAM placed into brown vials and stored frozen in a desiccator.

7.12.2 ADAM working reagent (1 mg/mL):

The required number of vials are removed from the desiccator and 1.0 mL methanol added to each.

7.13 Chloroform/hexane (1:1):

Equal volumes of CHCl₃ and hexane.

7.14 Chloroform/methanol (95:5):

10 mL of methanol added to 190 mL of CHCl₃.

7.15 Mobile phase (routine):

Solvent A: Acetonitrile:water (70:30) - 700 mL acetonitrile + 300 mL GDW
Solvent B: Acetonitrile

7.16 Mobile phase (confirmatory):

Solvent A: Acetonitrile
Solvent B: Water
8. PROCEDURE

8.1 OA and DTX-1 extraction procedure.

8.1.1 Accurately weigh one gram of the digestive gland puree into a glass culture tube with a Teflon-lined screw cap.

8.1.2 Add 4.0 mL of methanol and blend thoroughly.

8.1.3 Centrifuge the sample at 1000 rpm for 10 minutes.

8.1.4 Pipet 2.5 mL of the methanollic extract into a glass culture tube with a Teflon-lined screw cap and add 0.5 mL of GDW.

8.1.5 Add 2.5 mL of hexane to the tube and mix the phases thoroughly with a vortex mixer and centrifuge to clear the phases. Draw off and discard the hexane and repeat.

8.1.6 Add 1.0 mL of GDW to the aqueous extract.

8.1.7 Extract the mixture with 4.0 mL of CHCl₃ and mix thoroughly with the vortex mixer, and centrifuge to clear the phases.

8.1.8 Transfer the lower CHCl₃ layer to a screw-capped culture tube using a Pasteur pipette.

8.1.9 Repeat the CHCl₃ extraction (without further addition of water) and combine the CHCl₃ layers.

8.1.10 Evaporate the CHCl₃ layers to dryness under nitrogen and redissolve the residue in 500 µL of methanol.

8.2 Fluorescence reaction:

8.2.1 Preparation of ADAM solution (0.1%):

8.2.1.1 Remove the desiccator containing the 1 mg samples of ADAM reagent from the freezer and allow to come to room temperature before being opened.

8.2.1.2 Remove the required number of vials from the desiccator and return it to the freezer. One vial (containing 1 mg) is required for every 4 reactions to be done (including standards, blanks and samples).

8.2.1.3 Dissolve the reagent in 1.0 mL methanol and mix. The
diluted reagent is to be made fresh before each batch of reactions.

8.2.2 Reaction of standards:

8.2.2.1 Add an aliquot (20 µL) of the *Prorocentrum lima* extract working standard to an amber (1.5 mL) autosampler vial containing 20 µL of HDC working standard and evaporate to dryness under a stream of nitrogen in a N-evaporator with a water bath temperature of less than 40 °C.

8.2.2.2 Add 200 µL of ADAM reagent to the vial, cap tightly, mix and react in the dark for one hour at room temperature.

8.2.3 Reaction of samples:

8.2.3.1 Add an aliquot of each extract (100 µL, 0.1 g equivalent of digestive glands) to each of two amber vials containing 20 µL of HDC working standard.

8.2.3.2 To one vial from each pair add 20 µL *Prorocentrum lima* extract working standard as a spike.

8.2.3.3 Evaporate the samples to dryness and add 200 µL of ADAM reagent to the vial, cap tightly, mix and react in the dark for one hour at room temperature.

8.3 Cleanup of derivatized samples:

8.3.1 Condition the silica cartridges on the vacuum manifold by washing with methanol (4 mL), CHCl₃ (4 mL), and CHCl₃/hexane (1:1, 2 mL). Set the flow rates through the cartridges between 5 and 10 mL per minute.

8.3.2 Evaporate the methanol from the standards, blanks, samples and samples plus spikes with a stream of nitrogen.

8.3.3 Transfer the residue quantitatively with CHCl₃/hexane (1:1, 2 x 0.5 mL) to a conditioned cartridge.

8.3.4 Wash the cartridge with CHCl₃/hexane (1:1, 5 mL) and CHCl₃ (5 mL).

8.3.5 Elute the esters from the column with CHCl₃/methanol (95:5, 5 mL) into 15 mL tapered centrifuge tubes.

8.3.6 Evaporate the eluate to dryness with a stream of nitrogen.
and redissolve the residue in 100 µL of methanol.

8.3.7 Transfer the samples to amber, 500 µL, tapered microvials and cap the vials with Teflon-lined caps for HPLC analysis.

8.4 Routine HPLC analysis:

8.4.1 Equilibrate HPLC system at a column oven temperature of 35 °C with 100 % Solvent A flowing through the column at 0.5 mL/min. for 20 minutes.

HPLC conditions:
Mobile phase - Solvent A - 70 % Acetonitrile/GDW
Solvent B - Acetonitrile
Flow rate - 0.5 mL/min.
Detector - Excitation λ- 249 nm
           - Emission λ- 407 nm
Run time - 22-25 minutes
Injection volume - 5 µL

Gradient-

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent B (%)</th>
<th>Flow Rate (mL/min)</th>
</tr>
</thead>
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<td>0</td>
<td>0.5</td>
</tr>
<tr>
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<td>0.5</td>
</tr>
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</tr>
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<td>9.3</td>
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</tr>
<tr>
<td>25.0</td>
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</table>

8.4.2 Calibrate the system by at least two repeat injections of the *Prorocentrum lima* extract working standard.

8.4.3 Inject samples, spiked samples, blanks and reference materials onto the HPLC column and measure the resultant peak areas obtained from the integrator. Peaks are identified by comparison of retention times with the most recently run standards, directly or through the use of relative retention times using the internal standard, HDC.

8.5 HPLC Confirmation Column:

8.5.1 Equilibrate the HPLC system at 35 °C with 80 % solvent A flowing through the column at 1.1 mL/min. for 20 minutes.
HPLC conditions:
Mobile phase - Solvent A - Acetonitrile
Solvent B - GDW
Isocratic - 80 % Solvent A
Detector - Excitation λ- 249 nm
- Emission λ- 407 nm
Run time - 45 minutes
Injection volume - 10 µL

9. CALCULATIONS:

9.1 Measure peak areas of OA and DTX-1 in the standards and prepare a calibration curve of the amount of OA and DTX-1 per injection versus peak areas.

9.2 Calculate the total µg of DTX-1 or OA in one gram of the sample using the following formula:

\[ \text{µg DTX-1 or OA/g} = \frac{(\text{PA} \times D)}{\text{PAS} \times W} \]

Where:
- PA = peak area/injection
- PAS = peak area per µg DTX-1 or OA standard
- D = dilution factor
- W = weight of the original tissue extracted in grams

The value obtained is corrected for recovery using the HDC internal standard.

10. PRECISION AND ACCURACY

10.1 Data from replicate analyses of mussel digestive gland tissues containing DTX-1 near the alert level of 1.0 µg/g indicate the reproducibility is ± 1 %. Samples spiked with OA showed similar results. Variations of less than ± 10 % can be expected as the method approaches its detection limit.

10.2 Data from repeat analyses of mussel digestive gland tissues containing a range of DTX-1 concentrations is shown in Appendix A, Table 1. Levels obtained in the first analysis (A) compared with the second (B) give ratios (A/B) of, or very close to 1.

11. REMARKS

11.1 Representative chromatograms of a Prorocentrum lima working
standard run on the routine Superspher 100 column and on the confirmatory Lichrospher column are shown in Appendix A, Figures 1 and 2 respectively. A calibration curve for OA is shown in Figure 3 while the chromatogram in Figure 4 shows a typical mussel digestive gland extract with and without added OA and DTX-1.

11.2 The percentage recoveries of OA and DTX-1 from spiked mussel digestive gland can be expected to approach 100% with spike levels between 0.2 and 1.0 µg/g.

11.3 It is important that the chloroform being used is ACS grade, as other grades such as HPLC grade do not contain stabilizers, which may affect the polarity of the solvent. Slight changes in polarity may affect clean-up and recovery of OA and DTX-1.

11.4 Use of Prorocentrum lima as a working standard is not essential as commercially available standards such as MUS2 contain DTX-1 at a sufficient level to provide the retention time information that is required.

11.5 ADAM reagent solutions should be prepared fresh daily. The procedure for ADAM reagent verification is shown in Appendix A.

11.6 Samples containing significant OA or DTX-1 should be confirmed using the confirmatory column and by passing the extract through the procedure used for the clean up of "dirty" samples outlined in Appendix B.

11.7 Exercise care when substituting columns or cartridges from different manufacturers or when substituting different products from the same manufacturer as there is a considerable variation in the activity of the commercially available silica columns. Changes to the elution conditions may be required to obtain the desired results.

11.8 In situations where digestive glands cannot be separated from the meat or for some reason are unavailable, the meat may be used in its place. The alert level for the DSP toxins in the meat would be approximately five times less than in the digestive glands or 0.2 µg/g total for OA and DTX-1.
12. REFERENCES


APPENDIX A

1. ADAM Reagent Verification:

1.1 Add an aliquot (50 µL) of ADAM reagent to a solution of CHCl₃-5 % glacial acetic acid (5 mL). React the mixture for 10 minutes.

1.2 Dilute an aliquot (50 µL) with CHCl₃ (5 mL), vortex and allow the solution to sit for two minutes.

1.3 Prepare blanks by adding an aliquot of ADAM reagent (50 µL) to CHCl₃ (5 mL), react for ten minutes and dilute as above.

1.4 Read solutions on a fluorimeter using a suitable scale and an excitation wavelength of approximately 350 nm and an emission wavelength of approximately 440 nm.
**Table 1:** Replicate OA and DTX-1 analysis of mussel digestive gland tissue analyzed by the recommended method over a range of concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Estimate (µg/g)</th>
<th>Ratio A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract #1 (A)</td>
<td>Extract #2 (B)</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>DTX-1</td>
</tr>
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<tr>
<td>7</td>
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</table>
Figure 1. HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of a *Prorocentrum lima* working standard (5 µL) run on a Superspher 100 (12.5 cm x 2 mm i.d.) 3 µ, RP-18 column. Operating conditions: oven temperature 35 °C, flow rate 0.5 mL/min. Program: 70 % acetonitrile (1.0 min.) to 90 % acetonitrile (6.0 min.) and hold (2.9 min.). Begin a gradient to 100 % acetonitrile (0.4 min.) while increasing the flow to 0.8 mL/min., hold (8.7 min.), then return to initial conditions (1.0 min.).
Figure 2. HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of a Prorocentrum lima standard (10 µL) run on a Lichrospher (25 cm x 4 mm i.d.) RP-18, 5 µ, column. Operating conditions: oven temperature 35 °C, flow rate 1.1 mL/min., 80 % acetonitrile run isocratically (45 min.).
Figure 3. Calibration curve for okadaic acid run on the Superspher column. Conditions as in Figure 1.
Figure 4. HPLC fluorescence trace of a mussel digestive gland extract containing only the internal standard HDC and the same mussel extract spiked at a level of 0.76 µg/g OA and 0.10 µg/g DTX-1 run on a Superspher 100, RP-18 column. Conditions as in Figure 1.
APPENDIX B

Cleanup Of Persistent Interfering Materials From The Routine HPLC Analysis Of The Diarrhetic Shellfish Toxins Okadaic Acid And Dinophysis Toxin-1

1. Scope and Application

1.1 This procedure is applicable to molluscan shellfish samples containing materials that persistently interfere with the routine okadaic acid (OA) and dinophysis toxin-1 (DTX-1) HPLC fluorescence analysis procedure of the Canadian Food Inspection Agency. The method can also be used to assist in the confirmation of OA and DTX-1 contamination in all samples.

2. Principle of the Method

2.1 Although no human illnesses have been reported in Canada for shellfish containing these interfering materials, DSP bioassays of some samples have caused symptoms and deaths in the bioassay animals. The interference is most severe in late summer and early fall. HPLC/MS can be used to monitor these samples and as a confirmatory tool; however, most routine laboratories do not have the resources or technical capabilities to be able to purchase and maintain such a system. Therefore, an inexpensive and reliable confirmatory method is a necessity.

2.2 Samples containing interfering materials not removed during the normal cleanup are subjected to an additional cleanup step before routine HPLC analysis. Portions of the extracted and derivatized sample from the routine cleanup are run on a normal phase silica HPLC cartridge column, and fractions containing OA and DTX-1 are recovered and analyzed using conditions as described in the routine procedure.

3. Interferences

3.1 It is speculated that the interfering materials may be fatty acids as they can react with the ADAM reagent and will also be present in the organic extract. This might also explain the apparent seasonal nature of the interference.
4. **Apparatus**

4.1 Isocratic HPLC pump capable of providing reliable flows at 1.0 mL/minute.

4.2 Sample injection system capable of up to 100 µL injections.

4.3 Column oven: capable of maintaining the column temperature at 30 °C.

4.4 HPLC UV monitor, capable of measurement at a wavelength of 420 nm.

4.5 Data-handling system and recording device.

4.6 Column: Brownlee cartridge column system with a silica MP 10 cm x 4.6 mm i.d. column and a 3 cm x 4.6 mm guard column.

4.7 Fraction collector capable of taking fractions at 1.0 minute intervals.

5. **Reagents**

5.1 Chloroform (CHCl₃), ACS grade containing 0.8 % ethanol as a preservative.

5.2 Methanol, HPLC grade.

5.3 Okadaic acid (OA): Certified reference standard, NRC, Halifax.

5.3.1 Stock standard (0.1 µg/µL):

25 µg of standard diluted to 250 µL with methanol.

5.3.2 Working standard (5 ng/µL):

10 µL of stock standard diluted to 200 µL with methanol.

5.4 *Prorocentrum lima* extract working solution (675 µg OA/mL, 105 µg DTX-1/mL):

The solution is a concentrated extract from laboratory cultured *Prorocentrum lima*. The solution (in 95 % ethanol) contains both OA and DTX-1 and is standardized for OA against the certified reference standard OACS. DTX-1 for
the purposes of quantitation is considered to generate an equivalent response to OA. Commercial standards are also available and may be substituted.

5.5 Hyodeoxycholic acid (HDC), ACS grade.

5.5.1 Stock standard (0.1 mg HDC/mL):
10 mg HDC dissolved in 95 % ethanol diluted to 100 mL.

5.5.2 Working standard (5 ng HDC/µL):
0.5 mL of stock diluted to 10 mL with 95 % ethanol.

5.6 Mobile phase HPLC system:
1 % methanol in CHCl₃.

6. **Procedure**

6.1 Evaporate derivatized samples under nitrogen following their elution from silica SPE cartridges during the routine procedure.

6.2 Dissolve residue in 60 µL of CHCl₃ and inject 55 µL of the sample onto the HPLC silica column.

6.3 HPLC analysis: Equilibrate the columns at 30 °C with fresh mobile phase for 20 minutes:

   Mobile phase - 1 % methanol in CHCl₃
   Flow - 1.0 mL/minute
   Wavelength - 420 nm
   Run time - 20 minutes
   Post run - 5.0 minutes

6.4 Calibrate the instrument with an injection of derivatized *Prorocentrum lima* working standard.

6.5 Collect fractions of interest together, evaporate to dryness under N₂.

6.6 Redissolve the residue in 100 µL of methanol.

6.7 Inject 5.0 µL of each fraction of interest from samples, blanks, and spiked samples onto the routine Superspher 100, RP-18 column for analysis.
7. Remarks

7.1 A representative chromatogram of an extract of scallop digestive glands containing significant interfering material run on a silica column and monitored at 420 nm is shown in Figure 1. DSP toxins are eluted during the 3 to 6 minute period while the interfering materials are eluted during the 7 to 10 minute period. HDC elutes later in the run during the 15 to 19 minute period.

7.2 Figure 2 shows the scallop extract above with and without the additional cleanup, a spiked version of the same sample and the fraction containing the interfering material analyzed using the HPLC conditions as described in the routine procedure.

7.3 The percentage recoveries of OA and DTX-1 from the extended procedure can be expected to be in the range of 77 ± 10 % for OA and 70 ± 9 % for DTX-1.

7.4 ACS grade chloroform containing 0.8 % ethanol as a preservative should be used, otherwise the mobile phase conditions will have to be adjusted.

7.5 This procedure need only be employed if significant interferences are encountered or as a confirmatory tool if significant DSP contamination is suspected.
Figure 1. HPLC UV (420 nm) trace of a scallop digestive gland extract (55 µL) run on a Brownlee silica cartridge column 10 cm x 4.6 mm i.d. with a 3 cm x 4.6 mm i.d. guard column. Operating conditions: oven temperature 30 °C, flow rate 1.0 mL/minute, 1% methanol in chloroform run isocratically.
Figure 2. HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of a single derivatized scallop digestive gland extract run on a Supersphcr 100 (12.5 cm x 2 mm i.d.) 3 µ, RP-18 column. Section A: Routine procedure. Section B: DSP fraction from Brownlee column cleanup. Section C: DSP fraction from Brownlee column cleanup spiked with OA (0.76 µg/g) and DTX-1 (0.10 µg/g). Section D: Fraction from Brownlee column cleanup containing the interfering material. Operating conditions: oven temperature 35 °C, flow rate 0.5 mL/min. Program: 70% acetonitrile (1.0 min.) to 90 % acetonitrile (6.0 min.) and hold (2.9 min.). Begin a gradient to 100% acetonitrile (0.4 min.) while increasing the flow to 0.8 mL/min., hold (8.7 min.), then return to initial conditions (1.0 min.)