Extraction and Lipid Separation of Fish Samples for Contaminant Analysis and Lipid Determination

Standard Operating Procedure SOP No. HC521A

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1.0 Scope and Application

This method covers the extraction of fish samples for organic analysis by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS). For analysis of PCBs and the chlorinated pesticides currently reported by this laboratory (as of the above date), a 90/10 mixture by volume of petroleum ether/ethyl acetate is used for the extraction. If the samples are to be analyzed for general contaminants a 80/20 mixture of the same solvents is used. If more polar pesticides (such as atrazine) are analyzed by this laboratory in the future, a more polar extracting solvent mixture will probably be needed.

2.0 Summary of Method

This method covers only the extraction and cleanup portions of the testing procedure and is applicable to fish only. Analytical procedures for PCBs and pesticides in fish are already in place and are covered by the appropriate NBS/GLSC methods.

3.0 Interferences

3.1 Interferences from sample preparation glassware and reagents are routinely monitored by running method blanks. The method blank is run through the entire extraction process along with the samples, except that it consists only of sodium sulfate, the compound that is mixed with fish tissue before extraction.

3.2 All glassware is cleaned as soon as possible after use by rinsing with the last solvent used in it. Solvent rinsing is followed by detergent washing with hot water, and rinses with tap water and distilled water. The glassware is then drained dry and heated in a muffle furnace at 400°C for two hours. The glassware is solvent rinsed with acetone, hexane, and the solvent or solvent mixture used for a given operation immediately prior to glassware use. Volumetric ware is not heated in a muffle furnace. After cooling and drying, glassware is sealed and stored in a clean environment to prevent accumulation of dust and other contaminants. Glassware is stored inverted or capped with aluminum foil.

4.0 Safety

PCBs and pesticides have been tentatively classified as known or suspected, human or mammalian carcinogens. The toxicity or carcinogenicity of each chemical and reagent used in this method has not been precisely defined, although each chemical compound should be treated as a potential health hazard. The NBS Great Lakes Science Center maintains a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets is also available to all personnel involved in chemical analysis.

5.0 Apparatus

5.1 Glass chromatography columns -- 330 mm x 23 mm I.D., each fitted with a removable teflon stopcock. Kontes K-420540-0234 or equivalent.

5.2 Beakers -- 150 mL Pyrex Berzelius beakers.

5.3 Glass wool -- Corning Pyrex brand #3950 or equivalent. Solvents -- Petroleum ether, ethyl acetate, and iso-octane -- Pesticide quality or equivalent.

5.4 Zymark Turbovap II Concentrator.

5.5 Zymark Turbovap Concentration Tubes, 200 mL with 1 mL endpoint.

5.6 Zymark Benchmate Workstation robot equipped with Rheodyne 700 L switch valve with a 2 mL loop.

5.7 HPLC solvent pump, Waters, or equivalent.

5.8 ISCO Foxy 200 fraction collector.

5.9 Glass GPC column (51 x 1.5 cm i.d) packed with 200-400 mesh SX-3 biobeads.

5.10 Jones disposable 0.5 g micro silica gel columns.

5.11 Concentration Tubes, Kadurna-Danish - 10 mL. graduated. Kontes k-570050-1025 or equivalent.

5.12 Organomation N-Evap Concentrator Model III.

5.13 Argon gas, purified grade.

5.14 Vials, 15 mL glass, with Teflon-lined screw cap.

5.15 Sodium sulfate -- (ACS) Granular, anhydrous. Purified by heating at 400°C. for 4 h in a shallow tray. Supelco 2-0296 or equivalent.

6.0 Extraction Procedure

6.1 Sample Preparation

Samples are processed in sets of about 5-14 samples including all the necessary QA/QC samples specified in the QAPjP for this work. Fish tissue, previously homogenized according to the fish processing method HC500A.SOP, is thawed and thoroughly mixed. Ten g of tissue is weighed into a contaminant-free beaker and mixed with 40.0 g of Na2SO4 which has been previously dried by heating to 140°C overnight. The mixture is stirred frequently until it is dry and free flowing, containing no large lumps. The beaker is

then labelled with the appropriate sample number and weight. Necessary surrogates specified in the QAPjP Plan for this study.

6.2 Extraction

6.2.1 The extraction columns are prepared by inserting a small glass wool plug into the bottom of each chromatography column, and then the column is rinsed twice with 15 mL of petroleum ether. Air is removed from the glass wool by lightly tapping it with a clean glass stirring rod. A Zymark concentration tube is placed under each column and the appropriate labels are transferred to these tubes. The sample mixture is then poured into the column, after which 50 mL of the appropriate solvent is added to the sample beaker, stirred, and transferred to the column. The solvent is allowed to pass through the column, but as it begins to elute into the concentration tube, the stopcock is closed. At this point the column is lightly stirred with a glass rod to remove trapped air. Elution is then continued at the rate of 1-2 mL/min. until the solvent level reaches the beginning of the sample mixture.

6.2.2 Another 50 mL of the appropriate solvent mixture is added and elution is continued at the same rate. The columns are allowed to drain completely after the second 50 mL of solvent is added. The stopcock tips are rinsed with ethyl acetate to wash any residual lipid and analytes into the concentration tube.

6.3 Extract Concentration

The eluant is concentrated by placing the Zymark concentrator tubes in the Turbovap concentrator. The concentrator's water bath is kept at 40°C, and the argon sweep gas pressure is set to 10-12 psi with the Turbovap's control knob. Samples are concentrated to 1 mL or the minimum amount allowed by a sample's lipid content, whichever volume is greater. The residual solvent from this process should be mostly ethyl acetate. Each extract is then transferred to a graduated (or marked) culture tube and diluted to volume (10 or 20 mL depending on lipid and contaminant concentration) with ethyl acetate. The tubes are sealed with a teflon-lined screw cap and the labels are transferred to the appropriate tubes.

6.4 Lipid Determination

Prior to cleanup by gel permeation chromatography, a volume of sample extract equivalent to 1 g of tissue is pipetted into a preweighed (after acetone rinsing and drying) aluminum drying pan. Preweighing is to the nearest 0.1 milligram. The extract is allowed to evaporate under static conditions in a fume hood for 2 hours. The pan is again weighed to the nearest 0.1 milligram and the percent extractable lipid is computed as 100 x (1-weight of residual lipid).

7.0 Lipid Separation/Removal

7.1 The GPC column is initially calibrated by taking 5 mL fractions as the elution proceeds through the dump cycle, during which the lipids are routed to a waste bottle, and then through the collect cycle, during which the compounds of interest are routed to the appropriate collecting station. The endpoint of lipid removal is monitored by the gravimetric procedure detailed in Section 6.4. The starting point of compound collection is monitored by GC/GCMS analysis of the 5 mL fractions. The normal dump fraction is about 43 mL, followed by a collect fraction of 45 mL.

7.2 The status of the GPC column is monitored by either a pre- or post-run procedure for each batch extraction. While a batch is proceeding, or processing put on hold until the spike sample (run first in a batch) is brought from 45 to 50 mL and 2 μL of this are injected into a GC/EC to estimate recovery before advancing to the next sample. The peak height ratios of transnonachlor (elutes at beginning of collect cycle) and p,p'-DDE (elutes toward the end of collect cycle) should closely match that in the spike standard. The GPC can be prevented from advancing to the next sample or stopped at any point in the sample set. But once a sample is loaded on the column that sample will need to be rerun in case of a problem. If the original calibration conditions are changed after the spike sample any samples in the batch processed by GPC before correcting the problem must be rerun. Usually the GPC monitoring will not indicate a problem so frequently the spike will be checked during or after the entire batch has been processed or when recoveries are determined from previous sample sets to be low.

7.3 For PCB and/or pesticides analysis, a 2 mL portion of sample extract is loaded into the calibrated loop of the gel permeation system (attachment to the Benchmate Robot) and lipid/contaminants are separated with ethyl acetate through 200-400 mesh SX-3 Biobeads. The first fraction contains the lipids is discarded and the second fraction containing the contaminants is collected in a clean, appropriately labelled Zymark concentration tube. This fraction will be greater than 93% lipid-free. Consecutive loops of each extract can be processed when greater amounts of contaminants are needed (i.e. GC/MS). Refer to GPC technical manual for complete details of GPC setup and operation. The extracts are concentrated with the Zymark Turbovap set to the same conditions as specified in Section 6.3 above. The extracts are brought to 1 mL with the Zymark concentrator and then brought up to final volume with iso-octane.

7.4 Each sample is passed through a 0.5 g acidified micro-column of silica gel to remove the last 7% of the lipid. The silica gel column is designed to work with the Zymark Benchmate Workstation Robot. Fifty μL of 1:1 concentrated sulfuric and water is added manually to a silica gel column for each sample in the set being processed. Thereafter, the Benchmate will automatically rinse, load the sample (extract from 1 g of fish), weight amount loaded on the column, and elute the desired analytes from the column. Samples are then transferred to a graduated culture tube and evaporated using the Organomation N-Evap Concentrator to about 0.5 mL. The sample is then diluted to volume (usually 1-5 mL) with iso-octane and then transferred to a GC or GC/MS autosampler vial and stored until ready for analysis.

7.5 This is a manual method for removal of remaining lipids after GPC and was used for most of the mass balance fish samples beginning because with the automated method lipid was causing problems quantitating several PCB congeners. One mL of iso-octane is added to the GPC extract (equivalent to g fish tissue) and samples are concentrated to a final volume of 1 mL with the Zymark TurboVap set to conditions as specified in Section 6.3. Each sample is passed through a borosilicate glass column, 30 cm X 1.5 cm i.d., assembled as follows from bottom to top:

a. Pyrex wool plug
b. 1.0 g dried sodium sulfate
c. 7.0 g of a 10% (w/w) mixture sodium bicarbonate and sodium sulfate
d. 3.6 g 2% deactivated silica gel
e. 0.2-0.3 mL concentrated sulfuric acid

Each column is conditioned with 5 mL of the elution solvent (5:95 ethyl acetate:hexane) before loading sample onto the column. Zymark tubes are rinsed with 5 mL of elution solvent which is passed through the eluant from the column is collected in 12 mL calibrated centrifuge tube. Three additional 2 mL aliquots are passed through the column and collected in the same receiving tube. Each sample is then concentrated to the final volume necessary for analysis using a stream of argon gas, approx. 10 psi, and a water bath at 38°C.