Standard operating procedure for:

Extraction of Polycyclic Aromatic Hydrocarbons from Fish Tissue for Subsequent Analysis by GC/MS

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1. SCOPE AND APPLICATION

1.1 This method describes the extraction of polycyclic aromatic hydrocarbons (PAHs) from fish tissue.

1.2 This procedure can be used to isolate analytes listed in the following analysis SOP: SV-019-1 (Ref 14.1)

1.3 This procedure is to be used for all samples with the following Laboratory Information Management System (LIMS) test ID: T-PAH

1.4 The sample extraction is performed by using Dionex ASE-350 Accelerated Solvent Extraction System based on NOAA procedure (ref 14.2, 14.3). Concentration and GPC cleanup steps in this method are based on the EPA Method 3640A (see 14.4)

2. SUMMARY OF THE METHOD

2.1 Five (5) grams of ground fish tissue sample is mixed with Dionex ASE prep DE Diatomaceous Earth in a ASE350 extraction cell and left at room temperature to dry. The sample is then extracted in ASE 350 machine for a total time of 21 minutes using two extraction cycles. The extract is concentrated by nitrogen blowdown and brought to a volume of 4 mL or more.

2.2 The concentrated extract is then cleaned-up by passing through 10 g of silica and eluting with methylene chloride.

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2.3 The extract is subsequently concentrated to the volume of 1.0 mL (2x diluted with regard to the final method volume of 0.5 mL). 0.8 mL aliquot of extract is cleaned up with GPC and concentrated to 0.4 mL that is submitted for analysis. 100 uL of Internal Standards mix (at 400 ug/L) is added to the extract just before the final blowdown.

3. APPARATUS AND EQUIPMENT

3.1 IChem 60 mL jars with screw caps.

3.2 Beakers, 400 mL

3.3 Blender, 12 Speed, Osterizer

3.4 Blade, Teflon O-ring, and screw cap assemblies for blender

3.5 Ground glass stoppers to fit concentrator tubes

3.6 Top Loading Balance, accurate to 0.1 g Adventure SL, AS153 SN 8026461189.

3.7 Volumetric pipets, Class A - 0.50, 1.0, 2.0, 5.0, 10.0 mL.

3.8 Volumetric flasks, Class A - 10, 25, 50, 100, 200, 250, 500, 1000 mL.

3.9 Disposable Pasteur pipets.

3.10 Gas tight syringes - 50, 100, 250, 500, 1000 µL.

3.11 N-Evap®116(Organomation Associates, Inc): consists of heated water bath and apparatus to further concentrate the extract using nitrogen gas. The nitrogen should be passed through a drying filter to remove traces of water. It can concentrate 34 samples simultaneously.

3.12 Aluminum weighing pans, for dry weight determination. (only if required, i.e.: PT samples)

3.13 50-mL culture tubes.

3.14 Shimadzu HPLC SYSTEM for GPC clean-ups, consisting of - System controller, LC 20ADVP pump, Autosampler SIL-10AP or SIL-20A HT, UV detector and Fraction collector- ISCO Foxy 200 or Foxy R2. The GPC acquisition is controlled by EZchrom Software.

3.15 GPC Column: Phenomenex EnviroSep – ABC 60x21.2 mm (Guard column), Phenomenex EnviroSep ABC 350x21.2mm (Main column).

3.16 Gelman 0.45 µm syringe filters.

3.17 Sample vials - 1, 2 and 4 mL

3.18 UV detector - variable or fixed wavelength at 254 nm.

3.19 Centrifuge.

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3.20 High temperature muffle furnace – Thermo Scientific –Lindbergh Blue M.. All glassware used must be rinsed with tap water and DI water. Air-dry the glassware, and then bake in the oven 525 °C for 8 hours.

3.21 Oven- Fisher Scientific. ASE 350 extraction cells, silica, glasswool and diatomaceous earth are stored there at 120°C.

3.22 Knives.

3.23 Cutting board.

3.24 Saw blades.

3.25 Screw cup centrifuge tubes, 5 mL

3.26 Teflon lined screw cups for 5 mL centrifuge tubes

3.27 60 mL glass columns for silica cleanup procedure.

4. REAGENTS AND CHEMICALS

4.1 Solvents: methylene chloride - HPLC grade - Fisher Scientific.

4.2 Diatomaceous Earth (Dionex), granular, anhydrous. Purified by heating at 700 °C for 12 hours or overnight. It is then stored at 120°C in an oven.

4.3 GPC Calibration Solution - Prepare a solution in methylene chloride that contains the following compounds at the listed concentrations. See preparation details in GPC SOP GC-029-1 (see 14.5):

Analyte	**mg/mL**
Biphenyl	0.01
Perylene	0.01

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1 Fish tissue samples are collected in glass jars with Teflon-lined screw caps. The samples are maintained at <6°C from the time of collection until receipt at the laboratory, to a maximum time of 24 hrs. If a longer transport time is necessary, the samples are to be frozen. Ideally, fish should be frozen upon collection and shipped to the laboratory under dry ice. Tissue samples are frozen at the laboratory upon receipt and maintained in the dark at less than -10°C. (Ref 14.8, 14.9)

5.2 Samples must be extracted within 365 days of collection. The extract must be analyzed within 40 days after sample preparation.

6. SAMPLE HOMOGENIZATION

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6.1 Thoroughly clean all glassware by rinsing it with hot tap water and deionized water, then bake it in an oven at 525 °C for 8 hours. Anything that cannot be baked, including fillet knives, cutting boards, and blender blade assemblies should be thoroughly washed with Alconox and hot water, then rinsed with hot tap water, deionized water, and acetone. Subsequently all the mechanical parts are sonicated in acetone and dried. **Pre-rinse all glassware with methylene chloride prior to use!**

Due to the ubiquitous nature of Polycyclic Aromatic Hydrocarbons (PAHs) combined with very low detection limits of the Agilent 7000C GC/MS/MS system some level of contamination is always to be expected. Constant efforts have to be made by all the technicians and analysts to keep the background contamination levels as low as possible. It is highly recommended to keep all the low level PAH preparation work physically separated from high level PAH analysis. This especially pertains tothe oil fingerprinting/total hydrocarbons analyses that deal with very high concentrations and even neat petroleum products. Furthermore special precautions should be taken when using mechanical devices such as grinders during sample preparation. Mechanical devices use petroleum lubricants that easily contaminate tissue samples.

6.2 Check out the samples from the freezer.

6.3 When homogenizing fish, wash the knife and cutting board with Alconox, hot tap water, deionized water and acetone between samples. Change gloves between samples as well. Only use Diamond Grip latex gloves from Microflex due to possibility of cross-contamination with petroleum based residue often found on laboratory gloves. Diamond Grip gloves showed lowest levels of contamination in laboratory tests. The gloves, once on hands, should be washed with soap and water. When using the blender, use a different blade assembly (blade, O-ring, and screw cap) for each sample.

6.4 The amount of the fish sample taken for analysis is usually 5 g, but may be smaller if only fat is to be analyzed. Different preparation procedures are used based on test requirement:

6.4.1 If the whole fish is requested cut entire fish, including organs, into small pieces and blend them into a homogenous paste. Take a 5-g portion from each sample for preparation.

6.4.2 If only the fillet is to be analyzed, cut each fillet into small pieces and blend them into a paste. Take a 5-g portion from each sample for preparation.

6.4.3 If specific organs are to be analyzed (e.g., gonads, etc.), remove the organs, cut them into small pieces, and then blend them into a paste. For gonads, take a 5-g portion of each sample for preparation.

6.4.4 If fat only is to be analyzed, blend the fat from each sample into a paste. Take a 0.5 to 1g portion of each sample for preparation.

6.5 Unless a PAHs free tissue sample can be found and procured to be used as a clean matrix, the Method Blank (MBLK) and the Laboratory Fortified Blank (LFB) are prepared using Dionex Diatomaceous Earth. To prepare matrix spikes select one sample and weigh out three portions instead of one -- two of these will be used as duplicate Matrix Spikes. If none of the samples have enough tissue to prepare duplicate matrix spikes, prepare a single matrix spike or no matrix spike. In this case prepare duplicate LFBs. Make a note in the Preparation Log Book that there was not enough sample to prepare Matrix Spikes.

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6.6 After taking a portion of each sample for preparation, put the rest of each sample paste into a labeled 60 mL IChem jar. Cap the jars and store them in the tissue freezer at –20 °C or below.

6.7 Mix the sample portion that was taken for preparation with 5 g of Diatomaceous Earth in a labeled 60 mL IChem jar. Let samples sit for a while at room temperature to fully desiccate. Mix frequently to disperse the tissue. Once dried the samples are ready to be transferred into ASE350 extraction cell.

7. SAMPLE EXTRACTION

Tissue samples are analyzed in batches of up to 20 samples. In addition to the tissue samples in each batch there are typically one Method Blank, Laboratory Fortified Blanks (LFBs) and Matrix Spikes. Replicate tissue samples may also be analyzed in special cases.

NOTE: A SECOND PERSON MUST WITNESS THE ADDITION OF SPIKE STANDARD TO THE PROPER SAMPLES, VERIFY THE CORRECT VOLUME AND CONCENTRATION, AND ENSURE CORRECT RECORDING.

7.1 Clean the ASE 350 extraction cells.
ASE 350 extraction cells are cleaned by washing with Alconox and water. They are then rinsed with water, DI water and acetone followed by sonication in acetone and drying in the air. Empty cells are then placed in the ASE 350 machine and further cleaned by running the extraction program. After they are removed from the ASE 350 carousel they are stored in an oven at 120 °C.

7.2 Remove the appropriate surrogate and spiking solutions from the refrigerator. Let them warm to room temperature.

7.3 Clearly label the ASE extraction cells and place a glass-fiber filter at the bottom of each of them. Transfer the sample/drying agent mixture to the ASE cell (the ASE funnel may be used here). The bottom of the cell is tapped firmly but carefully on the counter top during the transfer to completely settle the cell contents. The remaining cell volume is then filled with diatomaceous earth.

7.4 Before proceeding, ensure that the spike and surrogate mixes are at room temperature. Many compounds easily precipitate out of solution when in the refrigerator or freezer and they need to be re-dissolved into solution by being warmed to room temperature and sonicated.

7.5 Spike all Samples, Blanks, and QC samples (Matrix Spikes, and Lab Fortified Blanks) with the 200 uL of PAH surrogate spike mix. Spike the LFB and Matrix Spikes with the appropriate PAH spiking solution (100 uL).

7.6 Clear the cell threads of sample/DE mixture if necessary. Cap the cell firmly but not forcefully.

7.7 The ASE cells and the ASE collection vials are then loaded in the corresponding positions in the ASE carousels. Collection vials for system rinses should be loaded in the rinse vial positions.

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7.8 Press the TRAYS button on the ASE panel to lock the carousel into the starting position. Press the RINSE button twice to flush the solvent lines.

7.9 The sample is extracted using an ASE schedule with the RINSE parameter set to [ON], and using a method with the parameter values set as follows:

PREHEAT [0] min
HEAT [5] min
STATIC [5] min
FLUSH% [115] vol
PURGE [180] sec
CYCLES [2]
PRESSURE [2000] psi
TEMPERATURE [100] C
SOL A [MeCl2] [75]%
SOL B [Hexane] [25]%
SOL C [other] [0]%
SOL D [other] [0]%

7.10 After the extraction has finished remove the ASE vials from the ASE carousel.

8. SAMPLE CONCENTRATION

8.1 Transfer the contents of each collection bottle into a labeled 50-mL culture tube using a disposable Pasteur pipet. Rinse the inside of the concentrator tube three times with 1 mL of methylene chloride, and add the rinsates to the centrifuge tube.

8.2 Set up N-Evap® 116 by turning on nitrogen gas, heater (30 °C bath temperature), and attaching clean needles.

8.3 Open valves for each needle and check for required flow of nitrogen with a blank solvent in a centrifuge tube. Let the nitrogen flow for 5 minutes to clean the lines.

8.4 Place concentrator tubes into N-Evap®116 tray, and then carefully pull needles down to approximately 2 inches from solvent level. Make sure N_2 flow is adequate, but be careful NOT to blow N_2 too vigorously into sample, causing splashing.

8.5 Concentrate and make up to 4 mL or other final volume depending of lipids content.

8.6 Proceed with clean up procedures.

9. CLEANUP PROCEDURES

9.1 All tissue samples should be cleaned by passing through 10g Silica Gel column followed by Gel Permeation Chromatography (GPC cleanup). For GPC CLEANUP find the procedure in the Lab SOP for GPC CLEANUP GC-029-1 (see 14.5).

9.2 10 g Silica Gel/5 g alumina cleanup

9.2.1 1-5 g silica/ may be used for clean samples including method blanks and LFBs

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9.2.2 Place 10 g of silica into 60 mL glass column plugged with glasswool and ½ inch layer of granular sodium sulfate. Cover with another ½ layer of granular sodium sulfate on the top. Carefully add enough methylene chloride to completely cover the silica layer. Wash the silica gel with additional 15 mL of l methylene chloride.

9.2.5 Transfer the concentrated extract (4mL) to the column and elute with 25 mL of methylene chloride.

9.2.8 Using Nitrogen blow-down system concentrate the eluent to 1 mL. 0.8 mL is then injected into a GPC system for further cleanup.

10. ELECTRONIC SAMPLE PREPARATION LOGS

Each sample preparation must be recorded by the preparation team in the electronic Prep Worksheet Manager module of LIMS. For PAHs in tissue analysis there is a ready- made, partially prefilled template in LIMS called SOP-SV-016. The template names are identical with LIMS prep ID.

Many fields in both the header and the grid are prefilled and seldom change. They need, however, to be always checked for correctness.

Header fields in the electronic preparation logs:

- **Worksheet ID#:** *this is assigned automatically by LIMS. Example SOP-SV-016_2*
- **Prep ID:** *LIMS prep ID defined in LIMS and connected with test ID. Each test ID may only be connected to one Prep ID, but many test IDs may use the same prep method.*
- **Prepared By**:
- **Prep Start Date/Time**:*This will be the prep date in LIMS reports*
- **Spike Witness**;
- **Prep Completion Date/Time**:
- **Date/Time Reviewed**:
- **Reviewed By**:
- **Equipment ID**: ASE 350 SN 100704459
- **Balance ID**: *pre-entered , usually the same*
- **Spike 1:** *Std tracker SN; this is usually surrogate*
- **Spike 2:** *Std tracker SN; this is the PAH spikes used*
- **Water bath temp:** *in deg C usually 30 C*
- **Solvent 1** *Methylene Chloride_ lot number*
- **Cleanup procedures:** *GPC File: e.i.:100914GPC_3*
- **Prep comments:** *enter other chemicals/supplies here with lot numbers; example DE lot#., Silica lot# etc.*
- **Internal standard:** *enter the ISTD information like this: ISTD SN: 53803 10uL, 10-10-2014 IC*

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Grid Fields in the electronic preparation Logs

Prep Batch ID: ***consecutive "P" number assigned by LIMS in the form of P123456. The same number has to be used for sample reporting in QC manager.*****Sample ID**: *the sample IDs are selected in LIMS when the worksheet is created; for QC samples those rows are typically pre-filled in the template*

- **Sample ID**; *the laboratory sample Id assigned in LIMS*
- **Test ID**: *The analysis requested for the sample.*
- **Matrix:** *matrix assigned in LIMS*
- **Sample wet Wt (g)/**: M*ass of the sample used for analysis e.g. usually 5g of tissue*

- **Final extract Volume (mL**: The volume of the sample extract after full concentration step; 0.5 mL.
  - **Clean-up procedure**: *The clean-up procedure used, if applicable. Write down anything done differently than SOP requirements.*
  - **Spike 1:** *volume of surrogate spike added*
  - **Spike 2:** *volume of the PAH spike added*

- **Comments:** *This section should include any observations regarding unusual appearance or odor of the sample, any dilutions made during preparations or any other remarks about the preparation*.

11. SAMPLE ANALYSIS PROCEDURE

See analysis SOPs SV-018-1 (ref 14.1)

12. QUALITY CONTROL

See QA/QC SOP GC-001-2 (ref 14.6)

13. SAFETY/HAZARDOUS WASTE MANAGEMENT

13.1 Wear safety glasses, gloves and a lab coat at all times while performing sample preparation! Methylene chloride will burn any skin it contacts; so wear nitrile gloves (Latex gloves will not stand up to methylene chloride.).

13.2 Handle all the sample extraction, solvent transfer, concentration, sample preparation, and clean up in a hood at all times. Avoid breathing methylene chloride or other solvent vapors. All extractions MUST be prepared in a fume hood.

13.3 Discard all methylene chloride waste in the appropriate Chlorinated Waste bottles. Keep chlorinated and non-chlorinated waste solvents separate. If mixed, record the volume of each and dispose with chlorinated waste.

13.4 Dispose of leftover and expired neat standards into a sealed, labeled container.

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13.5 Dispose of all the stock standards, working standards and used sample extracts into a labeled chlorinated waste container.

13.6 Dispose of all used GC vials containing standards or samples into a labeled chlorinated waste container.

13.7 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available.

13.8 A reference file of material data handling sheets is kept in the office area, across from the entrance to the tech room (room A316J). Refer to this file for any material you handle. Update this file upon receiving new chemicals or reagents. The MSDS for many chemicals can also be viewed on-line, at http://hazard.com/MSDS/

13.9 Primary standards of these toxic compounds should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be worn when an analyst handles high concentrations of these toxic compounds.

13.10 Dispose of all unwanted, broken glassware into a broken glassware disposal box. Inspect every piece of glassware. Do not use any that are chipped, cracked, etched, or scratched. Glassware with minor damage should be stored for repair.

13.11 See Safety-Waste SOP OG-001-3 (see 14.7) for further details.

14. REFERENCES

14.1 DEP SOP SV-018-1 Analysis of Polycyclic Aromatic Hydrocarbons (PAHs) in Fish T by using GC/MS/MS.

14.2 Extraction, clean-up and gas chromatography/mass spectrometry analysis of sediments and tissues for organic contaminants" NOAA technical Memorandum NMFS-NWFSC-59, March 2004.

14.3 Extraction, clean-up end gas Chromatography/Mass spectrometry Analysis of edible seafood for parent and Alkyl polycyclic aromatic Hydrocarbons in response to the Deewater horizon MC252 Oil spill Event. NOAA method , July 2010

14.4 EPA 3640A Gel-Permeation Cleanup: Test Methods for Evaluating Solid Wastes, Physical/Chemical Methods, SW-846; 3rd Edition, Update 4, January 3, 2008.

14.5 DEP SOP GC-029-1 Gel-Permeation Chromatography (GPC) Cleanup procedure for Chlorinated Pesticides and PCBs analysis.

14.6 DEP SOP GC-001-2 - Quality Assurance / Quality Control in the GC-Pesticides Laboratory.

14.7 DEP SOP OG-001-3 Safety and Waste Management Practices in the Organic Analysis Group (Volatile, Semi-Volatile and Pesticides).

14.8 EPA 1668C Chlorinated Biphenyl Congeners in Water, Soil, Sediment, Biosolids and Tissue by HRGC/HRMS; April 2010

14.9 EPA 1699 Pesticides in Water, Soil, Sediment, Biosolids and Tissue by HRGC/HRMS; December 2007

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APPENDIX 1

TROUBLESHOOTING A GPC INSTRUMENT

Along with routine sample cleanup, a fully trained analyst should be able to troubleshoot a GPC instrument for problems that arise in normal operations. All of the GPC instruments have service contracts, so always consider calling the technical service department of the company who manufactured your GPC. While you may not need the service engineer to repair the problem on site, they can be a tremendous source of valuable information and can often diagnose your problem over the telephone. Telephone numbers for each company, service department, can be found in each instrument "Maintenance Log book" or in the instrument manual itself. As always, whenever you have gone through a troubleshooting/ repair episode, record all information in the pertinent GPC Instrument Maintenance Logbook so information will be available for the next person when he or she (or you, again!) is attempting to diagnose a problem.

And as always, proper routine maintenance of the GPC can alleviate many major repairs in the future. Some basic maintenance points:

- Clean GPC columns after use by flushing with methylene chloride.
- After every 50-60 samples, the GPC column need back flush. It also depends how dirty are the samples run in the instrument.
- Back flush procedure for the GPC column: Connect the column in opposite direction. Flush the column with methylene chloride for 1h. Inject 1 mL of THF and run for 5 min, then inject another 1 mL of toluene and let it run for 30 min. Disconnect the column and connect in normal direction and flush the system for 30 min.
- Replace frits and seals routinely.
- Diagnose and fix high-pressure problems as they occur. Operating the HPLC at higher than normal pressures places stress on all the LC components and can contribute to a more rapid deterioration of components.
- Filter all samples that are injected into the LC.

APPENDIX OF CHANGES

12/18/2013	GPC calibration changed to biphenyl/perylene mix, neutral alumina added to the silica gel cleanup step.
12/05/2014	Addition of the Electronic Sample Preparation logs
11/13/2015	References to GC/MS TOF and GC/MS QQQ replaced with Agilent 5977 GC/MS
04/04/2016	Extraction solvent changed to CH2Cl2-hexane 3:1, removal of neutral alumina from the cleanup procedure, references to GC/MS-TOF and GC/MS SIM replaced with GC/MS/MS.

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