NOAA Technical Memorandum NMFS-NWFSC-125

Northwest Fisheries Science Center's Analyses of Tissue, Sediment, and Water Samples for Organic Contaminants by Gas Chromatography/Mass Spectrometry and Analyses of Tissue for Lipid Classes by Thin Layer Chromatography/ Flame Ionization Detection

January 2014

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
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Northwest Fisheries Science Center

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Northwest Fisheries Science Center's Analyses of Tissue, Sediment, and Water Samples for Organic Contaminants by Gas Chromatography/Mass Spectrometry and Analyses of Tissue for Lipid Classes by Thin Layer Chromatography/ Flame Ionization Detection

Catherine A. Sloan, Bernadita F. Anulacion, Keri A. Baugh, Jennie L. Bolton, Daryle Boyd, Richard H. Boyer, Douglas G. Burrows, David P. Herman, Ronald W. Pearce, and Gina M. Ylitalo

Northwest Fisheries Science Center
2725 Montlake Boulevard East
Seattle, Washington 98112

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Executive Summary

This document describes the analytical methods developed and currently used by the Environmental Chemistry Program in the Environmental Conservation Division of the Northwest Fisheries Science Center for the analysis of sediment, tissue, and water samples for part-per-billion concentrations (nanograms per gram or nanograms per milliliter) of selected polycyclic aromatic hydrocarbons and persistent organic pollutants, plus selected alkanes in water samples as needed. Detailed descriptions are presented for sample extraction, cleanup by silica/alumina columns and by size-exclusion high-performance liquid chromatography, and analysis by gas chromatography/mass spectrometry, as well as the calculation of analyte concentrations and important quality assurance measurements. Detailed descriptions are also given for measuring lipid classes in tissues, determining the percent total extractable content of tissues, and determining the percent dry weight of tissues and sediments. Information pertaining to the necessary laboratory supplies and instruments is provided. The step-wise procedures are presented in a descriptive manner, which facilitates the use of this document by other laboratories.

Acknowledgments

We thank the past Environmental Chemistry Program chemists for their expert contributions, in particular, Donald Brown and Margaret Krahn.

Abbreviations

ASE	Accelerated Solvent Extractor
CS	calibration standard
DOB	dibromooctafluorobiphenyl
GC	gas chromatograph
GC/MS	gas chromatography/mass spectrometry
HMB	hexamethylbenzene
HPLC	high-performance liquid chromatography
id	inside diameter
IS	internal standard
LOQ	lower limit of quantitation
MS	mass spectrometer
NIST	National Institute of Standards and Technology
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PER	perylene
POP	persistent organic pollutant
QA	quality assurance
RF	response factor
RRF	relative response factor
RSD	relative standard deviation
RT	retention time
SIM	selected-ion monitoring
SRM	Standard Reference Material
SS	surrogate standard
TCMX	tetrachloro-*m*-xylene
TCOX	tetrachloro-*o*-xylene
TLC/FID	thin layer chromatography/flame ionization detection
UV	ultraviolet

1. Introduction and Summary of Analytical Procedures

A wide range of environmental studies has included the analyses of sediments, tissues of aquatic organisms, and water for toxic contaminants such as chlorinated pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), collectively referred to as persistent organic pollutants (POPs) hereafter, and polycyclic aromatic hydrocarbons and sulfur-containing heterocycles, collectively referred to as polycyclic aromatic hydrocarbons (PAHs) hereafter. High-quality data with documented quality assurance (QA) measures are needed for such analyses in order to draw valid conclusions and reach appropriate decisions, for example, in damage assessment cases, for policy making, and to facilitate comparisons of the results with those of other studies. New techniques are continually sought and evaluated to further optimize the analytical accuracy, precision, sensitivity, robustness, efficiency, and safety. The Environmental Chemistry Program of the Northwest Fisheries Science Center (NWFSC) Environmental Conservation Division develops, validates, and uses advanced methods for analyzing marine samples for organic contaminants at part-per-billion concentrations (ng per g or ng per mL). As improvements to procedures are validated, they are incorporated into the analytical protocols used for a multitude of projects in which selected PAHs (Table 1 and Table 2) and POPs (Table 3) are quantitated in sediment, tissue, and water.

Table 1. PAH analytes.

Naphthalene	Retene
1-Methylnaphthalene	Fluoranthene
2-Methylnaphthalene	Pyrene
2,6-Dimethylnaphthalene	Benz[*a*]anthracene
Acenaphthylene	Sum of chrysene and triphenylene*
Acenaphthene	Benzo[*b*]fluoranthene
2,3,5-Trimethylnaphthalene	Sum of benzo[*j*]fluoranthene and benzo[*k*]fluoranthene*
Fluorene	Benzo[*e*]pyrene
Dibenzothiophene	Benzo[*a*]pyrene
Phenanthrene	Perylene
Anthracene	Indeno[1,2,3-*cd*]pyrene
1-Methylphenanthrene	Sum of dibenz[*a,h*]anthracene and dibenz[*a,c*]anthracene*
3-Methylphenanthrene	Benzo[*ghi*]perylene
9-Methylphenanthrene	
1,7-Dimethylphenanthrene	

* These analytes are quantitated and reported as the sum of their concentrations because they coelute during gas chromatography/mass spectrometry (GC/MS) analysis; the first analyte is present in the calibration standard, whereas the additional analyte is not.

Table 2. PAH analytes, including sums of alkyl PAH isomers.

Naphthalene
C1-Naphthalenes: sum of 1-methylnaphthalene and 2-methylnaphthalene
C2-Naphthalenes: sum of dimethyl- and ethyl-naphthalenes, including 2,6-dimethylnaphthalene[a]
C3-Naphthalenes: sum of trimethyl-, methylethyl- and propyl-naphthalenes, including 2,3,5-trimethylnaphthalene[a]
C4-Naphthalenes: sum of tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl-naphthalenes
Acenaphthylene
Acenaphthene
Fluorene
C1-Fluorenes: sum of methyl-fluorenes
C2-Fluorenes: sum of dimethyl- and ethyl-fluorenes
C3-Fluorenes: sum of trimethyl-, methylethyl- and propyl-fluorenes
Dibenzothiophene
C1-Dibenzothiophenes: sum of methyl-dibenzothiophenes
C2-Dibenzothiophenes: sum of dimethyl- and ethyl-dibenzothiophenes
C3-Dibenzothiophenes: sum of trimethyl-, methylethyl-, and propyl-dibenzothiophenes
C4-Dibenzothiophenes: sum of tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl-dibenzothiophenes
Phenanthrene
Anthracene
C1-Phenanthrenes and anthracenes[b]: sum of methyl-phenanthrenes and anthracenes, including 1-methylphenanthrene[a], 3-methylphenanthrene and 9-methylphenanthrene
C2-Phenanthrenes and anthracenes[b]: sum of dimethyl- and ethyl-phenanthrenes and anthracenes, including 1,7-dimethylphenanthrene[a]
C3-Phenanthrenes and anthracenes[b]: sum of trimethyl-, methylethyl-, and propyl-phenanthrenes and anthracenes
C4-Phenanthrenes and anthracenes[b]: sum of tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl-phenanthrenes and anthracenes
Retene
Fluoranthene
Pyrene
C1-Fluoranthenes and pyrenes[b]: sum of methyl-fluoranthenes and pyrenes
C2-Fluoranthenes and pyrenes[b]: sum of dimethyl- and ethyl-fluoranthenes and pyrenes
C3-Fluoranthenes and pyrenes[b]: sum of trimethyl-, methylethyl-, and propyl-fluoranthenes and pyrenes
C4-Fluoranthenes and pyrenes[b]: sum of tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl-fluoranthenes and pyrenes
Benz[*a*]anthracene
Sum of chrysene and triphenylene[c]
C1-Benz[*a*]anthracenes and chrysenes[b]: sum of methyl-benzanthracenes and chrysenes
C2-Benz[*a*]anthracenes and chrysenes[b]: sum of dimethyl- and ethyl-benzanthracenes and chrysenes
C3-Benz[*a*]anthracenes and chrysenes[b]: sum of trimethyl-, methylethyl-, and propyl-benzanthracenes and chrysenes
C4-Benz[*a*]anthracenes and chrysenes[b]: sum of tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl-benzanthracenes and chrysenes
Benzo[*b*]fluoranthene
Sum of benzo[*k*]fluoranthene and benzo[*j*]fluoranthene[c]

Table 2 continued. PAH analytes, including sums of alkyl PAH isomers.

Benzo[*e*]pyrene
Benzo[*a*]pyrene
Perylene
Indeno[1,2,3-*cd*]pyrene
Sum of dibenz[*a,h*]anthracene and dibenz[*a,c*]anthracene[c]
Benzo[*ghi*]perylene

[a] This alkyl PAH is used to calculate the GC/MS response factor for the quantitation of its group of isomers (also see Table 9).
[b] These analytes are quantitated and reported as the sum of their concentrations because these isomers co-occur, and in some cases coelute, in the same region of the GC/MS chromatogram.
[c] These analytes are quantitated and reported as the sum of their concentrations because they coelute during GC/MS analysis; the first analyte is present in the calibration standard, whereas the additional analyte is not.

This document incorporates the latest revisions to the methods described in Sloan et al. (2004, 2005) and Ylitalo et al. (2005), including the addition of quantitating PBDE analytes by gas chromatography/mass spectrometry (GC/MS). Among the additions is the method for quantitating the following lipid classes by thin layer chromatography/flame ionization detection (TLC/FID):

Wax esters and sterol esters,
Triglycerides,
Free fatty acids,
Cholesterol, and
Phospholipids and other polar lipids.

Also included is the method for analysis of water samples for PAHs and POPs plus the following selected alkanes as needed:

Pristane,
Phytane,
n-Heptadecane, and
n-Octadecane.

These detailed descriptions of our current procedures are provided for complete documentation as well as for ease of use by other laboratories.

Preparation and analyses of tissue and sediment samples are summarized in Figure 1. Weighed portions of sediment or tissue samples are mixed with sodium sulfate and magnesium sulfate, then extracted with dichloromethane using an Accelerated Solvent Extractor (ASE) (Dionex, Salt Lake City, UT). Extracts of tissues, such as marine mammal tissues, may be split into portions: one for isolating and quantitating the organic contaminants, one for quantitating lipid classes as needed, and one for determining the percent total extractables (i.e., mostly lipids) as needed. The extract or portion of extract to be analyzed for contaminants is filtered through a gravity-flow silica/alumina column. The filtered sample extracts are then concentrated, and a portion of each filtered extract is chromatographed on a size-exclusion high-performance liquid chromatography (HPLC) column, using dichloromethane as the mobile phase, to collect a fraction containing the PAHs and POPs. The dichloromethane is replaced with isooctane while

Table 3. Persistent organic pollutant analytes, including PCB congeners[a] and PBDE congeners.[b]

PCB 17	PCB 206
PCB 18	PCB 208
PCB 28	PCB 209
PCB 31	2,4'-DDD (*o,p'*-DDD)
PCB 33	4,4'-DDD (*p,p'*-DDD)
PCB 44	2,4'-DDE (*o,p'*-DDE)
PCB 49	4,4'-DDE (*p,p'*-DDE)
PCB 52	2,4'-DDT (*o,p'*-DDT)
PCB 66	4,4'-DDT (*p,p'*-DDT)
PCB 70	Aldrin
PCB 74	*cis*-Chlordane
PCB 82	*trans*-Chlordane
PCB 87	Dieldrin
PCB 95	Endosulfan I
PCB 99	Heptachlor
Sum of PCB 101 and PCB 90[c]	Heptachlor epoxide
PCB 105	Hexachlorobenzene
PCB 110	*alpha*-Hexachlorocyclohexane
PCB 118	*beta*-Hexachlorocyclohexane
PCB 128	*gamma*-Hexachlorocyclohexane (lindane)
Sum of PCB 138, PCB 163, and PCB 164[c]	Mirex
PCB 149	*cis*-Nonachlor
PCB 151	*trans*-Nonachlor
Sum of PCB 153 and PCB 132[c]	Nonachlor III
PCB 156	Oxychlordane
PCB 158	PBDE 28
PCB 170	PBDE 47
PCB 171	PBDE 49
PCB 177	PBDE 66
PCB 180	PBDE 85
PCB 183	PBDE 99
Sum of PCB 187, PCB 159, and PCB 182[c]	PBDE 100
PCB 191	PBDE 153
PCB 194	PBDE 154
PCB 195	PBDE 155
PCB 199	PBDE 183
PCB 205	

[a] PCB congeners are numbered according to Ballschmiter et al. 1992.
[b] PBDE congeners are numbered as is done for PCBs according to Ballschmiter et al. 1992.
[c] These analytes are quantitated and reported as the sum of their concentrations because they coelute during GC/MS analysis; the first congener is present in the calibration standard, whereas the additional congeners are not.

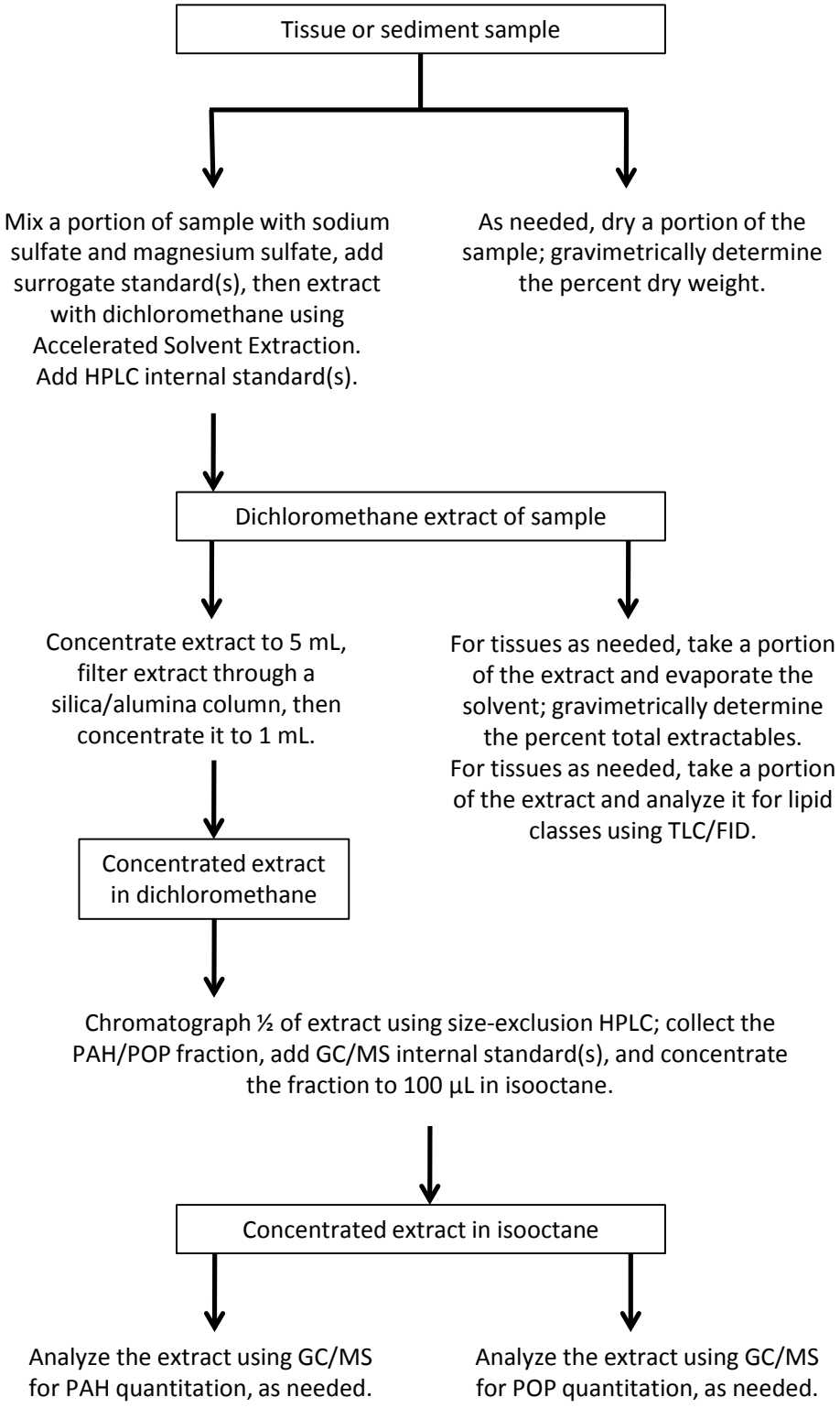

Figure 1. Flow diagram of tissue or sediment sample preparation and analysis.

the extracts are reduced to a final volume of approximately 100 µL each. The concentrated extracts are then analyzed by GC/MS to measure PAHs or POPs or both. Analytes are quantitated relative to surrogate standards (SSs), also known as extraction internal standards (ISs), using multiple concentration levels of GC/MS calibration standards (CSs). The concentrations of analytes in sediment and tissue samples are reported on a nanogram per gram (ng/g) wet weight or dry weight basis. The percent dry weight of tissues or sediments is determined gravimetrically using a separate portion of the unprocessed sample. The percent total extractables in tissues is determined gravimetrically using a portion of the extract taken prior to cleanup. Lipid classes are quantitated by TLC/FID using another portion of the tissue extract taken prior to cleanup.

Preparation and analyses of water samples are summarized in Figure 2. Measured volumes of water samples are extracted with dichloromethane using separatory funnels. The extract is cleaned up, prepared for GC/MS, and analyzed as is done for sediments and tissues, except that if the sample is to be analyzed for alkanes, the extract is not chromatographed on HPLC prior to being prepared for GC/MS and the alkanes are quantitated concurrently with the PAHs. The concentrations in water samples are reported in nanograms per milliliter (ng/mL).

Samples are typically analyzed in batches of 10 to 14 field samples. QA measures are incorporated into each batch according to the Environmental Chemistry Program's Quality Assurance Plan (Sloan et al. 2006). Each batch of samples includes a method blank, a sample of an appropriate National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) for tissues or sediment that has certified concentrations for many analytes, a spiked blank for water, and as needed, replicate samples. SSs are added to the samples before extraction to monitor and account for any losses during sample preparation. HPLC ISs are added after extraction, before any aliquots of the extract are taken (e.g., for percent total extractables determination), in order to account for the amount of total extract that is chromatographed by HPLC, and GC ISs are added before GC/MS analysis to measure the recovery of the HPLC ISs. Typical goals for QA measurements are given in Section 7.

Samples are prepared in fume hoods to minimize exposure of the analysts to solvent vapors, and personal protective equipment (e.g., nitrile gloves, safety glasses) is worn by the analysts whenever organic solvents are used. In some instances (e.g., for tissues and fluids of stranded marine mammals), samples may need to be weighed and prepared for the ASE in a biological safety cabinet to minimize exposure of the analysts to potential pathogens. Additional laboratory safety practices are followed according to the NWFSC Chemical Hygiene Plan and the NWFSC Chemical Waste Management Guide.

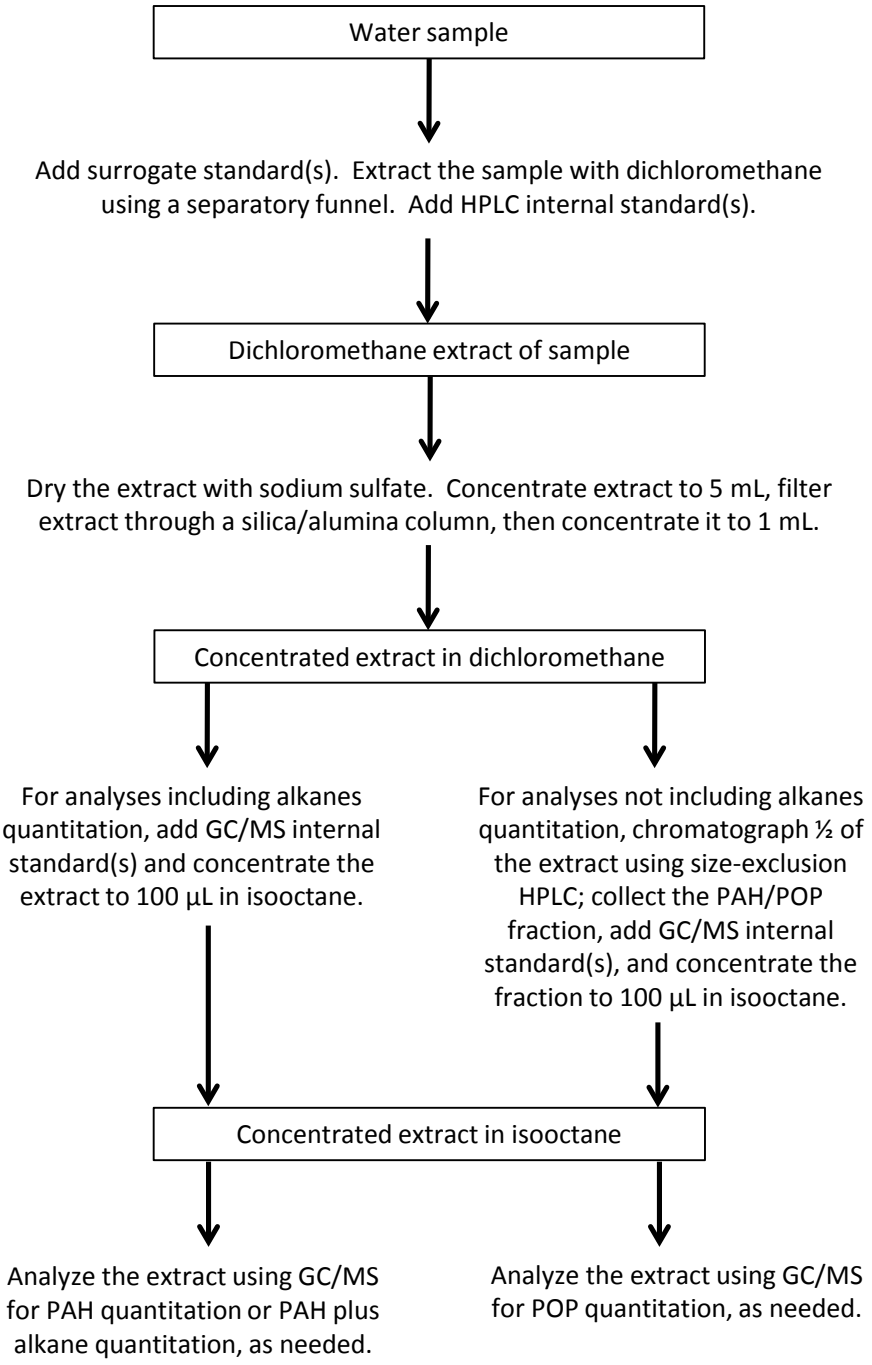

Figure 2. Flow diagram of water sample preparation and analysis.

2. Materials

2.1. Instruments and Accessories

2.1.1. GC/MS System

- Autosampler, Agilent 7693A, with on-column injection syringe and needle, and needle guide for 0.53-mm columns (Agilent Technologies, Wilmington, DE).
- Data system software, ChemStation, Version E (Agilent Technologies, Wilmington, DE).
- Gas chromatograph (GC) with cool on-column inlet fitted with inset and septum cap for 0.53-mm columns and syringe needles, Agilent 7890A (Agilent Technologies, Wilmington, DE).
- Mass spectrometer (MS), Agilent 5975C Mass Selective Detector (Agilent Technologies, Wilmington, DE).
- Sample vial tray, Agilent 7693 (Agilent Technologies, Wilmington, DE).
- Septum, GC inlet, bleed/temperature-optimized, 5 mm, 5183-4758 (Agilent Technologies, Wilmington, DE).

2.1.2. GC Gas and Accessories

- Helium, grade 5.0, ultra-high purity, 99.999% (Central Welding Supply, Marysville, WA).
- Alltech indicating oxygen trap, 4004 (Grace Davison Discovery Sciences, Deerfield, IL).
- Combination indicating oxygen/moisture/hydrocarbon trap, helium-specific, 21982 (Restek Corp., Bellefonte, PA).
- Regulator, two-stage, 400 series.
- Regulator, one-stage, 400 series.

2.1.3. GC Column and Accessories

- Capillary column cleaving tool, 23740-u (Supelco Inc., Bellefonte, PA).
- Column nut, GC inlet, 05921-21170 (Agilent Technologies, Wilmington, DE).
- Column nut, MS interface, 05988-20066 (Agilent Technologies, Wilmington, DE).
- Column union, Ultimate Union with UltiMetal Plus ferrules (Agilent Technologies, Wilmington, DE).
- Ferrule for installing column in GC inlet, UltiMetal Plus, 0.53 mm, G3188-27503 (Agilent Technologies, Wilmington, DE).
- Ferrule for installing columns in MS interface, 15% graphite: 85% Vespel, 1/16″, 5181-3308, no-hole, drilled in-house (Agilent Technologies, Wilmington, DE).

- Guard column, deactivated fused-silica, 10 m × 0.53 mm, 160-2535-10 (Agilent Technologies, Wilmington, DE).
- GC column, fused-silica, J & W Scientific DB-5, 60 m × 0.25 mm, 0.25-µm film thickness, 122-5062 (Agilent Technologies, Wilmington, DE).
- Electronic Leak Detector, 22839 (Restek Corp., Bellefonte, PA).

2.1.4. HPLC System and Accessories

- Autosampler, Waters 717 plus (Waters Corp., Milford, MA).
- Data system software, Empower (Waters Corp., Milford, MA).
- Filter, 2-µm particle size, 39-04-03524, in-line after autosampler (removed from inside autosampler) (Optimize Technologies Inc., Oregon City, OR).
- Fraction collector, Gilson FC 204 (Gilson Co., Middleton, WI).
- Helium for solvent degassing, grade 5.0, 99.999% (Central Welding Supply, Marysville, WA).
- Helium sparger for solvent reservoir, 10 µm, 25312 (Restek Corp., Bellefonte, PA).
- Regulator for helium tank, two-stage with stainless steel diaphragm.
- HPLC pump, 515 (Waters Corp., Milford, MA).
- Size-exclusion column, Envirosep ABC, 350 mm × 21.2 mm, 00W-3035-P0 (Phenomenex Inc., Torrance, CA).
- Size-exclusion guard column, Envirosep ABC, 60 mm × 21.2 mm, 03R-3035-P0 (Phenomenex Inc., Torrance, CA).
- Solvent inlet filter for solvent reservoir, 10 µm, 59277 (Supelco Inc., Bellefonte, PA).
- UV/VIS detector, Spectra 100 (Spectra-Physics, San Jose, CA).
- Valve, six-port, 7010 (Rheodyne Inc., Cotati, CA).

2.1.5. Sample Extractor and Accessories

- Accelerated Solvent Extractor, ASE 200 (Dionex, Salt Lake City, UT).
- Extraction cells, 33 cc, 048764 (Dionex, Salt Lake City, UT).
- Nitrogen, grade 4.8 (Central Welding Supply, Marysville, WA).

2.1.6. TLC/FID and Accessories

- Thin layer chromatography/flame ionization detector, Iatroscan MK-6 (Bioscan, Washington, DC).
- Data system software, Empower (Waters Corp., Milford, MA).
- TLC rods, Chromarod, silica gel, type S-4 (Shell-USA, Fredericksburg, VA).
- TLC rod holder, SD-5 (Shell-USA, Fredericksburg, VA).
- TLC rod spotting guide (Shell-USA, Fredericksburg, VA).
- TLC development tank, 6″ wide × 7.7″ high × 1.25″ deep (Shell-USA, Fredericksburg, VA).

- Filter paper, Whatman grade no. 1, 32″ diameter cut to 5.75″ wide × 7.35″ high (VWR Scientific, West Chester, PA).
- Syringe, Hamilton, 2 µL (Fisher Scientific, Pittsburgh, PA).
- Ultra-high-purity hydrogen, 5.0 grade (Central Welding Supply, Marysville, WA).

2.2. Solvents and Reagents

- Acetone (C_3H_6O), HPLC-UV grade (Pharmco-APER, Brookfield, CT).
- Copper, reagent grade, granular 20–30 mesh, 1720-05 (JT Baker, Phillipsburg, NJ).
- Dichloromethane (CH_2Cl_2), high purity, 300-4 (Burdick & Jackson, Muskegon, MI).
- Diethyl ether, 31685 (Sigma-Aldrich, Saint Louis, MO).
- Formic acid (CH_2O_2), 88%, A118P-100 (Fisher Scientific, Fair Lawn, NJ).
- Hexane (C_6H_{14}), capillary GC-GC/MS grade, GC215-4 (Burdick & Jackson, Muskegon, MI).
- Hydrochloric acid (HCl), concentrated, A144-212 (Fisher Scientific, Fair Lawn, NJ).
- Isooctane (C_8H_{18}), Optima, 0301-4 (Fisher Scientific, Fair Lawn, NJ).
- Magnesium sulfate ($MgSO_4$), anhydrous, M65-3 (Fisher Scientific, Fair Lawn, NJ).
- Methanol (CH_3OH), HPLC grade, MX0475-1 (EM Science, Darmstadd, Germany).
- Sodium sulfate (Na_2SO_4), reagent grade, anhydrous, 10–60 mesh, S1461 (Spectrum, Gardena, CA).

2.3. Silica/Alumina Column Packing Material

- Alumina, 80–200 mesh, 0537-01 (JT Baker, Phillipsburg, NJ).
- Glass beads, borosilicate, 3-mm diameter, 26396-630 (VWR, Brisbane, CA).
- Glass wool, Pyrex, 3950 (Corning Inc., Corning, NY).
- Silica, 100–200 mesh, S679-212 (Fisher Scientific, Fair Lawn, NJ).

2.4. Labware

Labware that contact samples or reagents are rinsed with acetone before use.

- Balances, analytical, readable to 0.001 g, 0.0001 g, and 0.001 mg.
- Boiling chips, Teflon, AW0919120 (All-World Scientific, Monroe, WA).
- Desiccator with desiccant.
- Glass rod, 1.6-cm diameter, 45-cm length.
- Graduated cylinders, 25 mL and 100 mL, Pyrex glass.
- Microdispensers and micropipettors, variable volume, Teflon tip, disposable glass capillary bores.
- Muffle furnace.
- Ovens, drying, 50°C, 60°C, and 120°C.

- Separatory funnels with stopcocks, 500 mL.
- Spatulas, stainless steel.
- Vial heater, with a wire holder to accommodate ASE vials and an aluminum vial rack.

2.5. Disposable Items

Items are one-use only. Glass items are heated in a muffle furnace at 400°C for 18 hours, then stored in glass containers at room temperature before use. Weighing pans are dried in an oven at 120°C overnight, then cooled in a desiccator for 30 minutes before use.

- ASE glass fiber filters, 047017 (Dionex, Salt Lake City, UT).
- ASE vials, 60 mL, with caps and septa (Dionex, Salt Lake City, UT).
- Chromatography column, plain, custom made, 22-mm id × 25-cm length (DJ's Glass, Morgan Hill, CA).
- GC vials, 1.5-mL, with caps and septa (SUN-SRI, Rockwood, TN).
- GC vials with limited volume inserts (approximately 200 µL), with caps and septa (Wheaton, Millville, NJ).
- HPLC vials, 4 mL, with caps, septa, and 1-mL inserts (SUN-SRI, Rockwood, TN).
- Jars, 10 ounce, 2.5-inch id mouth (Aaron Packaging, Kent, WA).
- Pipettes, transfer, Pasteur-style, 9″ and 5¾″.
- Weighing pans, aluminum, 28-mm id, 12577-081 (VWR, Brisbane, CA).
- Weighing pans, aluminum, 43-mm id, 180-5537 (All-World Scientific, Monroe, WA).
- Weighing pans, aluminum, 75-mm id, 25433-020 (VWR, Brisbane, CA).

2.6. Standard Solutions and GC Conditioning Solutions

Solutions are prepared commercially or in-house from commercial stock solutions using isooctane as the solvent, except the PAH/POP HPLC retention time (RT) standard and the lipid classes TLC/FID calibration standards, which are prepared using dichloromethane. These solutions are stored at −20°C. PAH and POP standards do not expire unless a problem occurs (e.g., evaporation). Concentrations given are approximate; actual concentrations will vary slightly from batch to batch of the solution. The GC CSs, SSs, and ISs are prepared such that the concentrations of the SSs and ISs in final sample extracts analyzed by GC/MS are approximately equal to the concentrations of the SSs and ISs in the GC CSs. GC conditioning solutions are used to simulate tissue or sediment samples during GC/MS performance verification.

2.6.1. Surrogate Standard and Internal Standard Solutions

- PAH SS (surrogate standard for quantitating PAH analytes)—contains naphthalene-d8, acenaphthene-d10, and benzo[*a*]pyrene-d12 at 1.7 ng/µL each compound.
- PAH HPLC IS (HPLC internal standard for quantitating the PAH SS)—contains phenanthrene-d10 at 1.6 ng/µL.

- PAH GC IS (GC internal standard for quantitating the PAH HPLC IS)—contains hexamethylbenzene (HMB) at 10 ng/µL.
- POP SS (surrogate standard for quantitating POP analytes)—contains PCB 103 and dibromooctafluorobiphenyl (DOB) at 1 ng/µL.
- POP HPLC IS (HPLC internal standard for quantitating the POP SS)—contains tetrachloro-*m*-xylene (TCMX) at 1 ng/µL.
- POP GC IS (GC internal standard for quantitating the POP HPLC IS)—contains tetrachloro-*o*-xylene (TCOX) at 1 ng/µL.

2.6.2. Calibration Standards

- PAH/POP HPLC RT standard (retention time standard for daily HPLC calibration)—contains DOB and perylene (PER) at 0.075 ng/µL each compound in dichloromethane.
- PAH GC/MS calibration standards—contain the PAHs in Table 1, except 9-methylphenanthrene, retene, triphenylene, benzo[*j*]fluoranthene and dibenz[*a*,*c*]anthracene, at the following concentrations in isooctane: Level 1 at 0.001 ng/µL each compound, Level 2 at 0.003 ng/µL each compound, Level 3 at 0.01 ng/µL each compound, Level 4 at 0.03 ng/µL each compound, Level 5 at 0.1 ng/µL each compound, Level 6 at 0.3 ng/µL each compound, Level 7 at 1 ng/µL each compound, Level 8 at 3 ng/µL each compound, and Level 9 at 10 ng/µL each compound. All levels contain naphthalene-d8, acenaphthene-d10, benzo[*a*]pyrene-d12, and phenanthrene-d10 at 0.5 ng/µL each compound, and HMB at 3 ng/µL in all levels.
- PAH plus alkanes GC/MS calibration standards—contain the PAHs in Table 1, except 9-methylphenanthrene, retene, triphenylene, benzo[*j*]fluoranthene and dibenz[*a*,*c*]anthracene, plus contain the alkanes listed in Section 1 (pristane, phytane, n-heptadecane, and n-octadecane) at the following concentrations in isooctane: Level 1 at 0.003 ng/µL each compound, Level 2 at 0.01 ng/µL each compound, Level 3 at 0.03 ng/µL each compound, Level 4 at 0.1 ng/µL each compound, Level 5 at 0.3 ng/µL each compound, and Level 6 at 1 ng/µL each compound. All levels contain naphthalene-d8, acenaphthene-d10, benzo[*a*]pyrene-d12, and phenanthrene-d10 at 0.5 ng/µL each compound, and HMB at 3 ng/µL in all levels.
- POP GC/MS calibration standards—contain the POPs in Table 3, except nonachlor III, PBDE 85, and as noted for coeluting PCBs, at the following concentrations in isooctane: Level 1 at 0.0003 ng/µL each compound, Level 2 at 0.001 ng/µL each compound, Level 3 at 0.003 ng/µL each compound, Level 4 at 0.01 ng/µL each compound, Level 5 at 0.03 ng/µL each compound, Level 6 at 0.1 ng/µL each compound, Level 7 at 0.3 ng/µL each compound, Level 8 at 1 ng/µL each compound, Level 9 at 3 ng/µL each compound, Level 10 at 10 ng/µL each *trans*-nonachlor and 2,4'-DDE only, and Level 11 at 50 ng/µL each *trans*-nonachlor and 2,4'-DDE only. All levels contain PCB 103, DOB, TCMX, and TCOX at 0.3 ng/µL each compound.
- Lipid classes TLC/FID calibration standards—contain lauryl stearate, triolein, oleic acid, cholesterol and L-α-phosphatidylcholine at the following concentrations in dichloromethane: Level 1 at 6.67 mg/mL each lauryl stearate, triolein, and oleic acid and 1.33 mg/mL each cholesterol and L-α-phosphatidylcholine; Level 2 at 5.0 mg/mL each lauryl stearate, triolein, and oleic acid and 1.0 mg/mL each cholesterol and L-α-phosphatidylcholine; Level 3 at 3.75

mg/mL each lauryl stearate, triolein, and oleic acid and 0.75 mg/mL each cholesterol and L-α-phosphatidylcholine; Level 4 at 2.5 mg/mL each lauryl stearate, triolein, and oleic acid and 0.50 mg/mL each cholesterol and L-α-phosphatidylcholine; Level 5 at 1.25 mg/mL each lauryl stearate, triolein, and oleic acid and 0.25 mg/mL each cholesterol and L-α-phosphatidylcholine; Level 6 at 0.63 mg/mL each lauryl stearate, triolein, and oleic acid and 0.13 mg/mL each cholesterol and L-α-phosphatidylcholine. Calibration standards are prepared from primary standards every 4-6 months to ensure that the standards have not decomposed or oxidized. Primary standards are prepared every 1–1.5 years at the following concentrations in dichloromethane: 10 mg/mL each lauryl stearate, triolein, and oleic acid and 2 mg/mL each cholesterol and L-α-phosphatidylcholine.

2.6.3. Spike Solutions

- POP spike solution—contains the POPs in Table 3, except nonachlor III, PBDE 85, and as noted otherwise for coeluting PCBs, at 0.3 ng/µL each compound in isooctane.
- PAH spike solution—contains the PAHs in Table 1, except 9-methylphenanthrene, retene, triphenylene, and dibenz[*a*,*c*]anthracene, at 0.2 ng/µL each compound in isooctane.
- Alkane spike solution—contains the alkanes listed in Section 1 (pristane, phytane, n-heptadecane, and n-octadecane) at 1 ng/µL each compound in isooctane.

2.6.4. GC Conditioning Solutions

- Sediment GC Conditioning solution—a composite of moderately contaminated sediment extracts that have been prepared for GC/MS analysis, as described in Section 3 and Section 4, in isooctane.
- Low-lipid Tissue GC Conditioning solution—a composite of moderately contaminated, low-lipid tissue extracts that have been prepared for GC/MS analysis, as described in Section 3 and Section 4, in isooctane.
- High-lipid Tissue GC Conditioning solution—a composite of moderately contaminated, high-lipid tissue extracts that have been prepared for GC/MS analysis, as described in Section 3 and Section 4, in isooctane.

2.7. Standard Reference Materials

SRMs are purchased from NIST, Gaithersburg, MD.

- SRM 1974c Organics in Mussel Tissue (*Mytilus edulis*)—analyzed for QA measurements in batches of low-lipid tissue samples.
- SRM 1941b Organics in Marine Sediment—analyzed for QA measurements in batches of sediment samples.
- SRM 1945 Organics in Whale Blubber—analyzed for QA measurements in batches of high-lipid tissue samples.
- SRM 1947 Lake Michigan Fish Tissue—analyzed for QA measurements in batches of fish tissue samples.

- SRM 1958 Organic Contaminants in Fortified Human Serum—analyzed for QA measurements in batches of marine mammal blood/serum/plasma samples.

2.8. Preparation of Silica/Alumina Column Packing Material and Other Reagents

2.8.1. Silica

The silica is heated at 700°C for 18 hours then stored at 120°C. It is allowed to cool to room temperature in a desiccator just prior to use.

2.8.2. Alumina

The alumina is heated at 120°C for 2 hours then allowed to cool to room temperature in a desiccator just prior to use.

2.8.3. Glass Wool and Glass Beads

The glass wool and glass beads are prepared for use (separately) by heating them at 400°C for 18 hours. They are stored in respective covered glass containers at room temperature until use.

2.8.4. Copper

Less than 1 hour before use, the copper is activated in a fume hood by placing it in a glass beaker, covering it with concentrated hydrochloric acid, stirring it with a glass rod, and allowing it to stand for 5 minutes. The copper is then washed three times with water, followed by three times with methanol then three times with dichloromethane. The copper is stored covered with dichloromethane until use to avoid contact with air.

2.8.5. Sodium Sulfate

The sodium sulfate is heated at 700°C for 18 hours then stored at 120°C. It is allowed to cool to room temperature in a desiccator just prior to use.

2.8.6. Magnesium Sulfate

The magnesium sulfate is heated at 400°C for 18 hours then stored at 120°C. It is allowed to cool to room temperature in a desiccator just prior to use.

2.9. Purity Testing

New brands of reagents are tested for purity before their initial use in sample analyses. Triplicate samples of the new brand of reagent are analyzed along with triplicate samples of the brand of reagent currently in use, for comparison. For solid reagents, triplicate solvent blanks are also analyzed to demonstrate whether any impurities found are from the reagent being tested or are from the solvent or laboratory. New glassware is used during purity testing. All

glassware and equipment contacting the sample are heated in a muffle furnace or rinsed with acetone before use.

2.9.1. Isooctane Purity Test

PAH GC IS (30 µL), POP GC IS (30 µL), and isooctane (100 µL) are added to a labeled GC vial with a limited volume insert, and the sample is mixed thoroughly.

The sample and GC/MS calibration standards are analyzed for PAHs and POPs using GC/MS selected-ion monitoring (SIM) as described in Section 5 for low-lipid tissues. The GC/MS responses of any impurities that are detected at the same retention times as analytes should be smaller than the corresponding analyte responses in the lowest level of GC/MS calibration standard.

2.9.2. Dichloromethane Purity Test

Dichloromethane (60 mL) is added to an ASE vial. Five boiling chips are added to the ASE vial containing the sample, and the sample volume is reduced to approximately 2 mL using a vial heater.

PAH GC IS (30 µL), POP GC IS (30 µL), and isooctane (150 µL) are added to the sample, and the sample is mixed thoroughly.

The sample is transferred to a labeled 4-mL HPLC vial, a boiling chip is added, and the sample volume is reduced to 100 µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

The sample is transferred to a labeled GC vial with a limited volume insert.

The sample and GC/MS calibration standards are analyzed for PAHs and POPs using GC/MS SIM, as described in Section 5 for low-lipid tissues. The GC/MS responses of any impurities that are detected at the same retention times as analytes should be smaller than the corresponding analyte responses in the lowest level of GC/MS calibration standard.

2.9.3. Sodium Sulfate Purity Test

Two glass-fiber filters are placed at the bottom of an ASE cell. Sodium sulfate (20 cc) is added to the labeled ASE cell. One glass-fiber filter is placed on top of the sodium sulfate in the cell.

The sample is extracted using the ASE as described in Section 3.

Five boiling chips are added to the ASE vial containing the sample, and the sample volume is reduced to approximately 2 mL using a vial heater.

PAH GC IS (30 µL), POP GC IS (30 µL), and isooctane (150 µL) are added to the sample, and the sample is mixed thoroughly.

The sample is transferred to a labeled 4-mL HPLC vial, a boiling chip is added, and the sample volume is reduced to 100 µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

The sample is transferred to a labeled GC vial with a limited volume insert.

The sample and GC/MS calibration standards are analyzed for PAHs and POPs using GC/MS SIM, as described in Section 5 for low-lipid tissues. The GC/MS responses of any impurities that are detected at the same retention times as analytes should be smaller than the corresponding analyte responses in the lowest level of GC/MS calibration standard.

2.9.4. Magnesium Sulfate Purity Test

The Magnesium Sulfate Purity Test is performed following the same procedure as for the Sodium Sulfate Purity Test in Subsection 2.9.3, except that magnesium sulfate (15 cc) is added to the labeled ASE cell instead of sodium sulfate.

2.9.5. Silica or Alumina Purity Test

A silica/alumina column is prepared by adding a 10- to 15-mm plug of glass wool to a chromatography column and tamping it with a glass rod, then adding alumina (10 cc), followed by silica (20 cc) and glass beads (5 cc).

A solution of methanol (10%) in dichloromethane (35 mL total) is slowly added to the column and allowed to drain into a waste container. Then to flush the methanol from the column, dichloromethane (35 mL) is slowly added to the column and allowed to drain into a waste container. Next the tip of the column is rinsed with dichloromethane.

A labeled ASE vial is placed under the column.

Dichloromethane (55 mL) is added to the column and allowed to drain into the vial.

Five boiling chips are added to the ASE vial containing the sample, and the sample volume is reduced to approximately 2 mL using a vial heater.

PAH GC IS (30 µL), POP GC IS (30 µL), and isooctane (150 µL) are added to the sample, and the sample is mixed thoroughly.

The sample is transferred to a labeled 4-mL HPLC vial, a boiling chip is added, and the sample volume is reduced to 100 µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

The sample is transferred to a labeled GC vial with a limited volume insert.

The sample and GC/MS calibration standards are analyzed for PAHs and POPs using GC/MS SIM, as described in Section 5 for low-lipid tissues. The GC/MS responses of any impurities that are detected at the same retention times as analytes should be smaller than the corresponding analyte responses in the lowest level of GC/MS calibration standard.

3. Sample Extraction

3.1. Accelerated Solvent Extraction of Sediment and Tissue Samples

Sediments or tissues (e.g., muscle, liver, blubber, plasma, gastroenteric contents) are customarily analyzed in batches of 12 to 14 field samples. In addition to the sediment or tissue samples in each batch, there are typically one method blank sample, one sample of an appropriate SRM, and as needed, replicates of a field sample. Standard Check Solutions are prepared as described below for each class of analytes. If either POPs or PAHs are not to be quantitated in the samples, all the internal standards in the samples and the Standard Check Solution for that class of analytes in the batch are omitted.

The typical amount of sediment or low-lipid tissue analyzed is ≤2 g to avoid overloading the ASE with water. The amount of high-lipid tissue analyzed is ≤0.5 g to avoid overloading the size-exclusion HPLC with lipids.

3.1.1. Sample Preparation

A 10-ounce jar, an ASE cell, and an ASE collection vial are labeled with the sample number.

For sediment samples, standing water is decanted from the original sample container, and the sample is stirred to homogenize it. All pebbles, shells, biota, and other extraneous material are discarded.

For tissue samples, the sample is homogenized in a glass jar (the original sample container is used if appropriate).

For a batch of tissue samples requiring Total Extractables Determination (Macro or Micro, Subsection 8.2) or Lipid Classes (Section 9) or both, the ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the “ASE Vial Weight.”

The sample is transferred to the labeled 10-ounce jar and weighed to the nearest 0.001 g. The weight is recorded as the “Sample Weight.”

Sodium sulfate (15 cc) is added to the sample in the jar and mixed thoroughly with the sample to absorb the water in the sample, which allows greater extraction efficiency of the organic compounds. To avoid clumping and hardening of the sodium sulfate, the sample is mixed immediately after adding the sodium sulfate to the jar. The sample is mixed until it appears dry to avoid generating excessive heat in the next step, which could volatilize analytes such as the naphthalenes.

For increased absorption of water from the sample, magnesium sulfate (15 cc) is then added to the sample in the jar and mixed thoroughly with the sample.

Two glass-fiber filters are placed at the bottom of the labeled ASE cell.

The mixture of sample and drying agent is transferred to the ASE cell using the ASE funnel. The bottom of the cell is tapped firmly but carefully on the countertop to completely settle the cell contents.

The remaining cell volume is filled with sodium sulfate. Again, the bottom of the cell is tapped firmly but carefully on the countertop to completely settle the cell contents, leaving approximately 3 mm of void volume at the top of the cell.

One glass-fiber filter is placed on top of the sodium sulfate in the cell and pressed in without overlapping the rim of the cell.

3.1.2. Surrogate Standards and Standard Check Solutions

The volumes of standards used depends on the amount of extract injected on GC/MS, which depends on the sample type and, in the case of tissues, the method of gravimetrically determining the percent mass of nonvolatile extractable material (Total Extractables Determination). Macro Total Extractables Determination uses one-third of the extract, whereas Micro Total Extractables Determination uses 1.5 mL of the extract in order to reduce the lower limit of quantitation (LOQ) for PAHs and POPs.

3.1.2.1. Batches of tissue samples requiring Macro Total Extractables Determination

PAH SS (150 µL) and POP SS (150 µL) are added onto the top filter in the cell, and then dichloromethane (approximately 1 mL) is added onto the top filter in the cell to rinse the internal standards into the cell.

The cell threads are cleared of sulfates, if present, and the cell is capped firmly but not forcefully.

A PAH Standard Check Solution is prepared by adding isooctane (50 µL) and PAH SS (150 µL) to a labeled GC vial.

A POP Standard Check Solution is prepared by adding isooctane (50 µL) and POP SS (150 µL) to a labeled GC vial.

3.1.2.2. Batches of sediment samples or tissue samples (except plasma) not requiring Macro Total Extractables Determination

PAH SS (75 µL) and POP SS (75 µL) are added onto the top filter in the cell, and then dichloromethane (approximately 1 mL) is added onto the top filter in the cell to rinse the internal standards into the cell.

The cell threads are cleared of sulfates, if present, and the cell is capped firmly but not forcefully.

A PAH Standard Check Solution is prepared by adding isooctane (25 µL) and PAH SS (75 µL) to a labeled GC vial.

A POP Standard Check Solution is prepared by adding isooctane (25 µL) and POP SS (75 µL) to a labeled GC vial.

3.1.2.3. Batches of plasma samples

PAH SS (40 µL) and POP SS (40 µL) are added onto the top filter in the cell, and then dichloromethane (approximately 1 mL) is added onto the top filter in the cell to rinse the internal standards into the cell.

The cell threads are cleared of sulfates, if present, and the cell is capped firmly but not forcefully.

A PAH Standard Check Solution is prepared by adding isooctane (25 µL) and PAH SS (40 µL) to a labeled GC vial.

A POP Standard Check Solution is prepared by adding isooctane (25 µL) and POP SS (40 µL) to a labeled GC vial.

3.1.3. ASE Operation

The ASE cell and the ASE collection vial are loaded in the corresponding positions in the ASE carousels. Collection vials for system rinses are loaded in the rinse vial positions.

The ASE system-rinse function is activated three times to flush the solvent lines.

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	PRESSURE [2,000] psi
HEAT [5] min	TEMPERATURE [100]°C
STATIC [5] min	SOL A [MeCl2] [100]%
FLUSH % [115] vol	SOL B [Other] [0]%
PURGE [180] sec	SOL C [Other] [0]%
CYCLES [2]	SOL D [Other] [0]%

After the extraction is completed, the ASE cell and the ASE collection vial are removed from the ASE carousel. The contents of the ASE cell are discarded appropriately.

3.1.4. Preparation of Extracts

3.1.4.1. Batches of tissue samples requiring Macro Total Extractables Determination

PAH HPLC IS (150 µL) and POP HPLC IS (150 µL) are added to the ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (150 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

POP HPLC IS (150 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the "ASE Vial w/Extract Weight."

The sample number is etched on the tab of a 75-mm id aluminum weighing pan.

The weighing pan is weighed to the nearest 0.001 g, and the weight is recorded as the "Pan Weight."

Approximately one-third of the sample in the ASE collection vial is transferred to the pan. The ASE vial is capped immediately afterward.

The ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the "ASE Vial w/out TE Extract Weight."

The pan containing one-third of the sample proceeds to Total Extractables Determination as described in Subsection 8.2.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.1.4.2. Batches of tissue (except plasma) samples requiring Micro Total Extractables Determination

PAH HPLC IS (75 µL) and POP HPLC IS (75 µL) are added to the ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (75 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

POP HPLC IS (75 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the "ASE Vial w/Extract Weight."

A 1.5-mL GC vial with label and cap is weighed to the nearest 0.0001 g, and the weight is recorded in the logbook as the "GC Vial Weight."

Approximately 1.5 ml of the sample in the ASE vial is transferred to the 1.5-mL vial using a Pasteur pipet. The ASE vial and the 1.5-mL vial are capped immediately afterward.

The 1.5-mL GC vial with the 1.5 mL of sample is weighed to the nearest 0.0001 g, and the weight is recorded as the "GC Vial w/TE Aliquot Weight" in the logbook.

The sample number is marked on a 28-mm id aluminum weighing pan.

The weighing pan is weighed to the nearest 0.001 mg using a 6-place balance, and the weight is recorded as the "Pan Weight."

The 1.5 mL aliquot of sample in the GC vial is poured into the weighing pan. The GC vial is rinsed with 0.2 mL of dichloromethane, and then this dichloromethane is added to the pan. This GC vial rinsing is repeated twice, with the dichloromethane added to the pan.

The pan containing 1.5 mL of the sample plus rinses proceeds to Total Extractables Determination as described in Subsection 8.2.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.1.4.3. Batches of plasma samples requiring Micro Total Extractables Determination

PAH HPLC IS (40 µL) and POP HPLC IS (40 µL) are added to the ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (40 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

POP HPLC IS (40 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the "ASE Vial w/Extract Weight."

A 1.5-mL GC vial with label and cap is weighed to the nearest 0.0001 g, and the weight is recorded in the logbook as the "GC Vial Weight."

Approximately 1.5 ml of the sample in the ASE vial is transferred to the 1.5-mL vial using a Pasteur pipet. The ASE vial and the 1.5-mL vial are capped immediately afterward.

The 1.5-mL GC vial with the 1.5 mL of sample is weighed to the nearest 0.0001 g, and the weight is recorded as the "GC Vial w/TE Aliquot Weight" in the logbook.

The sample number is marked on a 28-mm id aluminum weighing pan.

The weighing pan is weighed to the nearest 0.001 mg using a 6-place balance, and the weight is recorded as the "Pan Weight."

The 1.5 mL aliquot of sample in the GC vial is poured into the weighing pan. The GC vial is rinsed with 0.2 mL of dichloromethane, and then this dichloromethane is added to the pan. This GC vial rinsing is repeated twice, with the dichloromethane added to the pan.

The pan containing 1.5 mL of the sample plus rinses proceeds to Total Extractables Determination as described in Subsection 8.2.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.1.4.4. Batches of sediment samples or tissue (except plasma) samples not requiring Macro or Micro Total Extractables Determination

PAH HPLC IS (75 µL) and POP HPLC IS (75 µL) are added to the ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (75 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

POP HPLC IS (75 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.1.4.5. Batches of plasma samples not requiring Macro or Micro Total Extractables Determination

PAH HPLC IS (40 µL) and POP HPLC IS (40 µL) are added to the ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (40 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

POP HPLC IS (40 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.1.4.6. Batches of tissue samples requiring Lipid Classes Determination

Lipid Class Determination may be done in addition to Macro or Micro Total Extractables Determination after the corresponding steps in Subsection 3.1.4.1, Subsection 3.1.4.2, or Subsection 3.1.4.3.

The ASE collection vial is weighed to the nearest 0.001 g, and the weight is recorded as the "ASE Vial w/Extract Weight," unless this has been done previously for Macro or Micro Total Extractables Determination.

A 1.5-mL GC vial with label and cap is weighed to the nearest 0.001 g, and the weight is recorded in the logbook as the "Iatro Vial Weight."

Approximately 1 ml of the sample in the ASE vial is transferred to the 1.5-mL vial using a micropippetor. The ASE vial and the 1.5-mL vial are capped immediately afterward.

The 1.5-mL GC vial with the 1 mL of sample is weighed to the nearest 0.001 g, and the weight is recorded as the "Iatro Vial w/Extract Weight" in the logbook.

The sample in the 1.5-mL vial proceeds to Lipid Classes determination as described in Section 9.

The sample in the ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

3.2. Liquid-liquid Extraction of Water Samples

Water samples are customarily analyzed in batches of 10 samples. In addition to the water samples in each batch, there are typically one method blank sample and one spiked blank sample (using project-specific control water for both), one laboratory solvent blank sample (no water), and as needed, one or more replicate water samples. Standard Check Solutions are prepared as described below for each class of analytes. If either POPs or PAHs are not to be quantitated in the samples, all the internal standards and the Standard Check Solution for that class of analytes are omitted. Alkanes are quantitated concurrently with PAHs and therefore require the PAH internal standards and the PAH Standard Check Solution.

Typically, the amount of water analyzed is 200 mL, depending on the expected level of contamination. Water samples are contained in 250-mL amber bottles. Dichloromethane is added (10% of volume of water, e.g., 20 mL of dichloromethane for 200 mL of water) as soon as possible after collection to inhibit biological activity.

PAH SS (200 µL) and POP SS (200 µL) are added to each water sample in its bottle.

For the spiked water sample, PAH Spike (200 µL) and POP Spike (200 µL) are also added to the sample in its bottle. If the alkanes are to be quantitated, Alkane Spike (200 µL) is also added.

The laboratory solvent blank is prepared by adding dichloromethane (40 mL), PAH SS (200 µL), and POP SS (200 µL) to a labeled ASE vial. This sample is set aside until the other samples have been extracted as described below.

A PAH Standard Check Solution is prepared by adding PAH SS (200 µL) and PAH Spike (200 µL) to a labeled GC vial.

A POP Standard Check Solution is prepared by adding POP SS (200 µL) and POP Spike (200 µL) to a labeled GC vial.

If the alkanes are to be quantitated, an Alkane Standard Check Solution is prepared by adding PAH SS (200 µL) and Alkane Spike (200 µL) to a labeled GC vial.

The water sample bottle is inverted several times to mix the sample, and then (with the bottle right-side up) the cap is slowly opened to vent the sample. The sample is poured into a separatory funnel (stopcock closed) on a ring stand, and the funnel is capped.

Dichloromethane (20 mL) is added to the sample bottle, the bottle is capped, and the dichloromethane is swirled in the bottle to rinse the bottle. The bottle is set aside until after the first extraction of the sample as described below.

While keeping the cap securely on the separatory funnel, the funnel is shaken a few times to mix the contents. With the funnel upside-down, the stopcock is slowly opened to vent the sample, and the stopcock is closed. This shaking and venting of the sample is repeated for 2 minutes to thoroughly mix and extract the sample.

The separatory funnel is set on a ring stand, and the water and dichloromethane phases are allowed to separate.

After the phases have separated in the separatory funnel, the lower (dichloromethane) phase is drained into a labeled ASE vial, leaving behind any emulsion.

The dichloromethane in the sample bottle is added to the water in the separatory funnel.

The extraction and phase separation are repeated as above, and the lower (dichloromethane) phase is drained into the labeled ASE vial, leaving behind any emulsion.

The water remaining in the separatory funnel is disposed of appropriately.

The laboratory solvent blank sample is treated the same as all other samples from this point on.

Sodium sulfate (15 cc) is added to the sample in the ASE vial to absorb any water present, the vial is capped, and the sample is mixed thoroughly. The sample is allowed to sit overnight.

The extract in the ASE vial is decanted slowly into a second labeled ASE vial, leaving the sodium sulfate in the first vial. Any extract remaining in the sodium sulfate is transferred to the vial using a pipette.

Dichloromethane (approximately 3 mL) is added to the first ASE vial (that had contained the extract) and swirled in the vial to rinse the vial, and then it is slowly decanted into the second ASE vial, leaving the sodium sulfate in the first vial. Any solvent remaining in the sodium sulfate is transferred to the second vial using a pipette.

The rinsing of the first ASE vial and the decanting into the second ASE vial are repeated as above.

PAH HPLC IS (200 µL) and POP HPLC IS (200 µL) are added to the second ASE collection vial, and the contents of the vial are mixed thoroughly.

PAH HPLC IS (200 µL) is added to the PAH Standard Check Solution and the Alkane Standard Check Solution, and each solution is mixed thoroughly.

POP HPLC IS (200 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

The sample in the second ASE collection vial proceeds to Sample Cleanup by Silica/Alumina Column Chromatography as described in Section 4.

4. Sample Cleanup

4.1. Silica/Alumina Column Chromatography

A gravity-flow silica/alumina column is used for removing extraneous polar compounds from the sample.

4.1.1. Calibration of Silica/Alumina Column

Calibration is performed once per lot of silica and once per lot of alumina before their use in sample analyses.

A spiked blank is prepared by adding dichloromethane (5 mL), POP SS (60 µL), PAH SS (60 µL), POP Spike (80 µL), and PAH Spike (80 µL) to a labeled ASE vial.

A POP Standard Check Solution is prepared by adding POP SS (60 µL) and POP Spike (80 µL) to a labeled GC vial.

A PAH Standard Check Solution is prepared by adding PAH SS (60 µL) and PAH spike (80 µL) to a labeled GC vial.

A silica/alumina column is prepared by adding a 10- to 15-mm plug of glass wool to a chromatography column and tamping it down with a glass rod, then adding alumina (10 cc), followed by silica (20 cc) and glass beads (5 cc).

The column packing is slightly deactivated for better PAH recovery by slowly adding a solution of methanol (10% by volume) in dichloromethane (35 mL total solution) to the column and allowing the solvent to drain into a waste container. Then to flush the methanol from the column, dichloromethane (35 mL) is slowly added to the column and allowed to drain into a waste container. The tip of the column is then rinsed with dichloromethane.

A second ASE vial labeled “F1” is placed under the column. The spiked blank is slowly decanted into the column, and the eluant is collected in the second ASE vial.

The first ASE vial, containing the spiked blank, is washed with dichloromethane (approximately 5 mL), the washings are slowly decanted into the column, and the eluant is collected in the second ASE vial. This step is repeated twice more.

Dichloromethane (35 mL) is added to the column, and the eluant is collected in the second ASE vial. The ASE vial containing the F1 fraction is then set aside.

A third ASE vial labeled “F2” is placed under the column. Dichloromethane (10 mL) is added to the column, and the eluant is collected in the third ASE vial.

Five boiling chip are added to each of the ASE vials containing the F1 and F2 fractions, and each fraction is concentrated to approximately 2 mL using a vial heater.

PAH GC IS (60 µL), POP GC IS (60 µL), and isooctane (200 µL) are added to each fraction, and each fraction is mixed thoroughly.

POP GC IS (60 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

PAH GC IS (60 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly.

Each fraction is transferred to a labeled 4-mL HPLC vial, a boiling chip is added, and the fraction is concentrated to 200 µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

Each fraction is transferred to a labeled GC vial with a limited volume insert.

Each fraction and Standard Check Solution is analyzed for PAHs and POPs using GC/MS SIM as described in Section 5 to compare the recoveries of the analytes and internal standards in the F1 and F2 fractions to those in the Standard Check Solutions. The analytes and internal standards should completely elute in F1 and none in F2. If any analytes elute in F2, then the volume of dichloromethane for eluting the PAH/POP fraction in Subsection 4.1.2 must be increased from 35 mL to 45 mL.

4.1.2. Sample Cleanup by Silica/Alumina Column Chromatography

Five boiling chips are added to the ASE collection vial containing the sample extract from Section 3, referred to as the first vial in this subsection, and the extract is concentrated to 5 mL using a vial heater.

A silica/alumina column is prepared by adding a 10- to 15-mm plug of glass wool to a chromatography column and tamping it down with a glass rod, then adding alumina (10 cc), followed by silica (20 cc) and glass beads (5 cc).

The column packing is slightly deactivated for better PAH recovery by slowly adding a solution of methanol (10% by volume) in dichloromethane (35 mL total solution) to the column and allowing the solvent to drain into a waste container. Then to flush the methanol from the column, dichloromethane (35 mL) is slowly added to the column and allowed to drain into a waste container. The tip of the column is then rinsed with dichloromethane.

A second labeled ASE vial is placed under the column. The sample extract in the first ASE vial is slowly decanted into the column, and the eluant is collected in the second ASE vial.

The inside of the first vial is washed with dichloromethane (approximately 5 mL), the washings are decanted into the column, and the eluant is collected in the second ASE vial. This step is repeated twice more.

Dichloromethane (35 mL) is added to the column, and the eluant is collected in the second ASE vial.

Five boiling chips are added to the ASE vial containing the sample eluted from the silica/alumina column, and the sample volume is reduced to approximately 2 mL using a vial heater.

The sample is transferred to a labeled 4-mL HPLC vial, a boiling chip is added, and the sample volume is reduced to approximately 1 mL using the vial heater.

For a batch of sediment samples, activated copper is added to the HPLC vial a few grains at a time until no further discoloring of the copper occurs. The HPLC vial is capped and stored overnight.

4.1.3. Preparation of Concentrated Extracts

4.1.3.1. Batches of sediment or tissue (except plasma) samples or water samples not analyzed for alkanes

The sample is transferred to an HPLC vial with a 1-mL insert (leaving the copper in the 4-mL HPLC vial in the case of sediment samples), and the volume brought to 1 mL either by adding dichloromethane or by evaporating the excess under a gentle stream of nitrogen gas.

The sample in the vial proceeds to HPLC as described in Subsection 4.2.

4.1.3.2. Batches of plasma samples

The sample is transferred to a GC vial and the volume brought to 0.35 mL under a gentle stream of nitrogen gas.

The sample is transferred to an HPLC vial with a 0.35-mL insert.

The sample in the vial proceeds to HPLC as described in Subsection 4.2, injecting 350 µL of sample (the entire sample).

4.1.3.3. Batches of water samples analyzed for alkanes

Approximately one-half of the sample is transferred to a GC vial, capped, and set aside in case additional cleanup by size-exclusion HPLC is needed for PAH or POP analyses (prior to size-exclusion HPLC, this aliquot is prepared as is done for plasma samples in Subsection 4.1.3.2). Note that the alkanes cannot be measured in samples cleaned up by size-exclusion HPLC.

PAH GC IS (100 µL), POP GC IS (100 µL), and isooctane (100 µL) are added to the remaining sample in the 4-mL vial, and the sample is mixed thoroughly.

PAH GC IS (200 µL) is added to the PAH Standard Check Solution and to the Alkane Standard Check Solution, and each solution is mixed thoroughly.

POP GC IS (200 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

A boiling chip is added to the remaining sample in the 4-mL vial, and the sample volume is reduced to 100 µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil. Samples known to have high concentrations of analytes may need to be diluted with additional isooctane.

The sample is transferred to a labeled GC vial with a limited volume insert.

The sample in the GC vial insert and the PAH Standard Check Solution are analyzed by GC/MS for PAHs as described in Section 5. Samples and an Alkane Standard Check Solution may be analyzed for alkanes concurrently with PAHs, as described in Subsection 5.2.2.

The sample in the GC vial insert and the POP Standard Check Solution are analyzed by GC/MS for POPs as described in Section 5.

4.2. Size-exclusion High-performance Liquid Chromatography

Size-exclusion HPLC is used for removing extraneous high-molecular-weight compounds from the sample. The HPLC system is programmed according to Table 4. The fraction-collection time is determined immediately before chromatographing a batch of samples, as in Subsection 4.2.3, using the results from the system calibration performed in Subsection 4.2.2.

4.2.1. Size-exclusion HPLC Start-up

A 4-L bottle of dichloromethane is installed in the solvent reservoir holder, and the solvent inlet filter and helium diffuser are placed at the bottom of the bottle. The dichloromethane is degassed for a minimum of 10 minutes prior to HPLC system operation by diffusing helium into the dichloromethane. The helium flows through a hydrocarbon trap and an oxygen trap installed in-line between the helium tank and the diffuser.

The purge valve on the pump is opened, and the pump is started in purge mode to purge the pump with the degassed solvent for 5 minutes, with the solvent flowing to the waste collection bottle. The purge valve is closed after the pump purge has finished.

Table 4. Size-exclusion HPLC events.

Approx. time (min)	HPLC module	Activity
<0	Autosampler	Sample is loop loaded with 500 µL of sample or calibration standard.
0	Data system	Signal is sent to autosampler to inject sample and to fraction collector to start timer.
Continuous	Pump	Isocratic mobile phase flows at 5 mL/min.
1	Autosampler	Injection port is rinsed; needle is rinsed.
14–23	Fraction collector	Fraction is collected.
25	Computer	Data system ends integration.

The pump is started with the flow rate of the solvent set at 1 mL/min for purging the autosampler, and the autosampler purge mode is started. If the autosampler compression test fails, the autosampler purge is repeated.

The pump flow is set to 5 mL/min, and the system pressure is monitored for stability.

The wavelength on the detector is set at 254 nm, and the baseline of the ultraviolet (UV) signal is zeroed.

The data system is programmed and initiated so that the PAH/POP HPLC RT standard is injected and chromatographed according to Table 4, except that the fraction collector is set for no collection.

A 4-mL vial is filled with the PAH/POP HPLC RT standard and placed in the autosampler carousel.

The PAH/POP HPLC RT standard is chromatographed three or more times, as necessary, to obtain three consecutive analyses having retention time stability to within +0.05 minutes of each other for 4,4'-dibromooctafluorobiphenyl (DOB, first peak) and for perylene (PER, last peak). It may be necessary to re-zero the baseline of the UV signal at approximately 10 minutes into the first analysis of the PAH/POP HPLC RT standard.

For each analysis of the RT standard, the system pressure and the retention times of DOB and PER are recorded.

When the retention times of the DOB and PER peaks in the RT standard have stabilized for three consecutive analyses, the system is ready to proceed with the System Calibration described in Subsection 4.2.2 or the Daily Calibration described in Subsection 4.2.3, using the retention times of the DOB and PER peaks to determine the fraction collection times.

4.2.2. Size-exclusion HPLC System Calibration

The HPLC system calibration is performed only when any component of the HPLC system (e.g., pump, column, length of tubing, etc.) is changed.

The HPLC is started, and the PAH/POP HPLC RT standard is chromatographed three or more times, as necessary, as described in Subsection 4.2.1.

Using the retention times in the last chromatogram of the PAH/POP HPLC RT standard, the fraction collector is programmed to collect 10 consecutive 0.1-minute fractions starting 1 minute before the retention time of DOB, and to collect 15 consecutive 0.1-minute fractions starting at the retention time of PER.

The fraction collector is configured with a tray for 60-mL ASE vials. Twenty-five 60-mL ASE vials labeled F1 through F25 are positioned in the fraction collector tray.

An HPLC system calibration solution is prepared by adding dichloromethane (900 µL) and PAH/POP HPLC RT standard (100 µL) to a 1.5-mL GC vial, then thoroughly mixing the

contents of the vial. The contents are transferred to a 4-mL HPLC vial with a 1-mL insert. This vial is positioned in the autosampler tray.

The autosampler is programmed and initiated so that the HPLC system calibration solution is injected and chromatographed according to the times in Table 4, except that the fractions are collected as programmed above. The retention times of DOB and PER are recorded.

When the analysis is finished, the pump, autosampler, and fraction collector are stopped and the detector is turned off.

The ASE vials containing fractions F1 through F25 are removed from the collector tray, and the remaining HPLC system calibration solution is removed from the autosampler.

POP GC IS (30 µL) and isooctane (150 µL) are added to each of the vials containing fractions F1 through F10, and the contents of each vial are mixed thoroughly and transferred to a labeled 1.5-mL GC vial.

PAH GC IS (30 µL) and isooctane (150 µL) are added to each vial containing fractions F11 through F25, and the contents of each vial are mixed thoroughly and transferred to a labeled 1.5-mL GC vial.

POP GC IS (30 µL), PAH GC IS (30 µL), and isooctane (120 µL) are added to the remaining HPLC system calibration solution, and the solution is mixed thoroughly and transferred to a labeled 1.5-mL GC vial.

A small boiling chip is added to each GC vial containing the fractions and the remaining HPLC system calibration solution, and the contents are reduced to 100 µL each using a vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

The remaining HPLC system calibration solution and each fraction are transferred to a labeled GC vial with a limited volume insert.

The fractions F1 through F10, the remaining HPLC system calibration solution, and the Level 6 POP GC/MS calibration solution are analyzed by GC/MS for POPs as described in Section 5. The Level 6 POP GC/MS calibration solution is used to check the reproducibility of the GC/MS for DOB relative to TCOX. The POP GC IS in the fractions is used to show that each fraction is injected and analyzed properly by the GC/MS.

The GC/MS chromatograms for fractions F1 through F10 are inspected to find the first fraction in which DOB appears with an area ≥1% of the DOB area in the remaining HPLC system calibration solution. The starting time for the collection of this fraction from the HPLC system is designated as the starting time (t1) of the DOB elution from the HPLC system (see Figure 3).

To allow for possible retention time drift, 0.2 minute is subtracted from t1 to obtain t2. Then t2 is subtracted from the DOB HPLC retention time (DOB RT) in the analysis of the HPLC system calibration standard to obtain t3 (see Figure 3).

The value for t3 is recorded for future use in the daily calibration of the HPLC (Subsection 4.2.3).

The fractions F11 through F25, the remaining HPLC system calibration solution, and the Level 5 PAH GC calibration standard are analyzed by GC/MS SIM for PAHs as described in Section 5. The Level 5 PAH GC calibration standard is used to check the reproducibility of the GC/MS for PER relative to HMB. The PAH GC IS in the fractions is used to show that each fraction is injected and analyzed properly by the GC/MS.

The GC/MS chromatograms for fractions F11 through F25 are inspected to find the last fraction in which PER appears with an area that is ≥1% of the PER peak area in the remaining HPLC system calibration solution. The ending time for the collection of this fraction is designated as the ending time (t5) of the PER elution from the HPLC system (see Figure 3).

To allow for possible retention time drift, 0.5 minute is added to t5 to obtain t6. Then the PER HPLC retention time (PER RT) in the analysis of the HPLC system calibration standard is subtracted from t6 to obtain t7 (see Figure 3).

The value for t7 is recorded for future use in the daily calibration of the HPLC (Subsection 4.2.3).

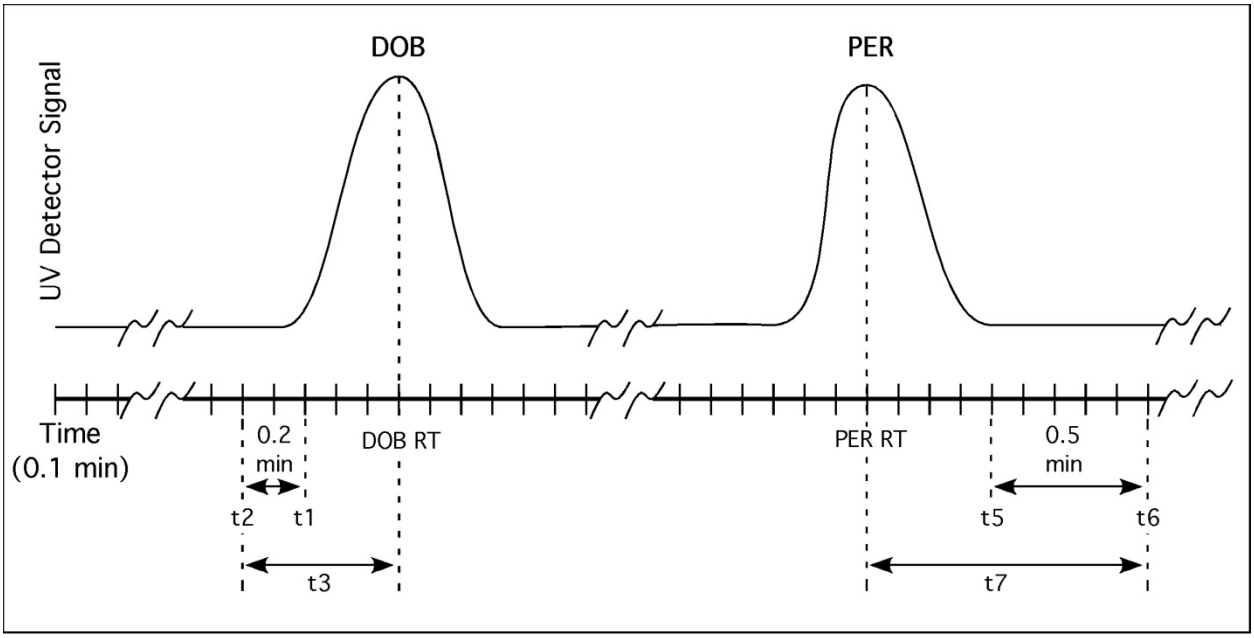

Figure 3. Size-exclusion high-performance liquid chromatograph calibration.

4.2.3. Daily Calibration of the Size-exclusion HPLC System

The daily calibration of the HPLC system is performed immediately before chromatographing a batch of samples to determine the collection time of the PAH/POP fraction.

The HPLC is started, and the PAH/POP HPLC RT standard is chromatographed three or more times, as necessary, as described in Subsection 4.2.1.

Collection start and end times for the PAH/POP fraction are determined using the retention times of DOB and PER in the most recent analysis of the PAH/POP HPLC RT standard, and the values t3 and t7 are derived from the HPLC system calibration described in Subsection 4.2.2.

For the daily calibration, the formula for the starting time t4 for collecting a PAH/POP fraction is

$$t4 = \text{DOB RT} - t3 \quad (1)$$

where DOB RT is the DOB HPLC retention time in the most recent analysis of the PAH/POP RT standard and t3 is the value determined in Subsection 4.2.2.

For the daily calibration, the formula for the ending time t8 for collecting a PAH/POP fraction is

$$t8 = \text{PER RT} + t7 \quad (2)$$

where PER RT is the PER HPLC retention time in the most recent analysis of the PAH/POP RT standard during the daily calibration and t7 is the value determined in Subsection 4.2.2.

4.2.4. Sample Cleanup by Size-exclusion HPLC

A batch of samples is chromatographed immediately following the daily calibration of the HPLC system described in Subsection 4.2.3, except for plasma samples the injection volume is 350 µL.

The fraction collector is programmed to collect the PAH/POP fraction for each sample in the batch, using the start and end times (t4 and t8, respectively) determined during the daily calibration of the HPLC system described in Subsection 4.2.3. The start and end times of the collection are recorded in the HPLC logbook.

Labeled ASE vials are placed in the rack of the fraction collector.

An HPLC Blank sample is prepared by filling a labeled 4-mL vial with dichloromethane. This sample is monitored for contamination from the HPLC system.

The batch of samples from Subsection 4.1.3, the HPLC Blank, and the PAH/POP HPLC RT standard are positioned in the autosampler tray such that the HPLC Blank is chromatographed at the beginning of the sequence and the PAH/POP HPLC RT standard is analyzed in the middle and at the end of the batch.

The data system is programmed to sequentially chromatograph the samples in the batch plus the PAH/POP HPLC RT standard in the middle and end of the batch.

For each analysis of the RT standard, the system pressure and the retention times of DOB and PER are recorded. If the retention time for DOB in the middle analysis differs from the first analysis by ±0.05 minute or more, then the starting time for collection of the fraction is changed accordingly for the remaining samples in the batch. If the retention time for PER in the middle analysis changed by ±0.05 minute or more with respect to the retention time for DOB in the middle analysis, then the collection time for the fraction is changed accordingly for the remaining samples in the batch.

After the sequence of samples and PAH/POP HPLC RT standard has finished, the guard column is backflushed for 15 minutes. Then the pump, autosampler, and fraction collector are stopped, and the detector is turned off.

The remaining sample in the HPLC vial insert is transferred to a GC vial and stored in a freezer as a reserve if needed.

4.2.5. Preparation of Extracts and Standard Check Solutions

Five boiling chips are added to the ASE vial containing the cleaned up extract, and the extract volume is reduced to approximately 2 mL using a vial heater.

For a batch of tissue, sediment, or water samples to be analyzed for both PAHs and POPs, PAH GC IS (30 µL), POP GC IS (30 µL), and isooctane (150 µL) are added to the ASE vial, and the contents are mixed thoroughly.

For a batch of tissue, sediment, or water samples to be analyzed for PAHs only, PAH GC IS (30 µL) and isooctane (175 µL) are added to the ASE vial, and the contents are mixed thoroughly.

For a batch of tissue, sediment, or water samples to be analyzed for POPs only, POP GC IS (30 µL) and isooctane (175 µL) are added to the ASE vial, and the contents are mixed thoroughly.

For the check solutions in a batch of tissue samples requiring Macro Total Extractables Determination: PAH GC IS (150 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly; POP GC IS (150 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

For the check solutions in a batch of sediment or water samples or tissue samples not requiring Macro Total Extractables Determination: PAH GC IS (75 µL) is added to the PAH Standard Check Solution, and the solution is mixed thoroughly; POP GC IS (75 µL) is added to the POP Standard Check Solution, and the solution is mixed thoroughly.

For all batches of sediment, tissue, or water, the sample is transferred from the ASE vial to a labeled 4-mL HPLC vial, a boiling chip is added, and the sample volume is reduced to 100

µL using the vial heater. All dichloromethane should be evaporated from the sample, indicated by the remaining isooctane ceasing to boil.

The sample is transferred to a labeled GC vial with a limited volume insert.

The sample and the appropriate Standard Check Solution are analyzed by GC/MS for PAHs or POPs as described in Section 5.

5. Gas Chromatography/Mass Spectrometry

GC/MS is used to measure the amounts of the analytes and the internal standards in the samples. The compounds are separated by GC and detected by MS, producing a chromatogram for each analysis, where the detector's response to the compounds produces peaks. The area under each peak is proportional to the concentration of the compound in the sample or standard analyzed.

5.1. GC/MS Operating Conditions

The operating conditions for the GC/MS are listed in Table 5.

Table 5. GC/MS operating conditions.

Injection volume:	2 µL
Injection technique:	cool on-column
Inlet temperature program:	oven temperature plus 3°C
Transfer line temperature:	320°C
Carrier gas:	helium
Carrier gas flow:	1.3 mL/min, constant flow
Column	
Material:	fused-silica capillary tubing
Length:	60 m
Internal diameter:	0.25 mm
Stationary phase:	DB-5
Phase composition:	5% phenyl, 95% methylpolysiloxane
Film thickness:	0.25 µm
Guard column	
Material:	deactivated fused-silica capillary tubing
Length:	10 m
Internal diameter:	0.53 mm
Quadrupole temperature:	150°C
Ion-source pressure:	$<5 \times 10^{-5}$ Torr
Ion-source temperature:	300°C
Ionization mode:	electron ionization
Filament:	chemical-ionization type (accommodates 300°C)
Tune mode:	Standard Tune; then the electron multiplier voltage is increased to achieve the desired sensitivity
Emission current maximum:	35 µA

The helium flows through a combination oxygen/moisture/hydrogen-indicating trap installed in-line between the helium regulators and the GC. In addition, an indicating oxygen trap is installed in-line at the rear of the GC.

The GC/MS is checked for atmospheric leaks, and the MS is tuned or checked for stability prior to analyzing a sequence of samples and standards.

5.2. GC/MS for Quantitating PAHs, Alkyl PAH Isomers, and Alkanes

The GC oven temperature is programmed for PAHs as follows.

Initial temperature:	80°C
Initial time:	1 min
First rate:	10°C/min to 200°C
Isothermal hold:	0 min
Second rate:	4°C/min to 300°C
Isothermal hold:	12 min at 300°C
Third rate:	10°C/min to 315°C
Isothermal hold:	15 min at 315°C
Total run time:	66.5 min

Sediment samples are analyzed using scan mode, scanning from 60 to 300 daltons. Tissue samples and water samples are analyzed using SIM mode, scanning only the quantitation and confirmation ions during the specified time window as shown in Table 6 for the analytes in Table 1 or as shown in Table 7 for the analytes in Table 2. See Subsection 5.2.2 for the alkane analytes. Approximate time windows are shown; see below for determining actual start and end times. Scan mode provides greater ability to confirm analyte identification in the complex sediment samples, whereas SIM mode generally provides greater sensitivity for tissue or water samples, which typically contain lower concentrations of contaminants.

5.2.1. GC/MS SIM Windows for PAHs and Alkyl PAH Isomers

For SIM analyses using Table 6, the actual time for ending a given window and starting the next window is determined as approximately the midpoint between the retention time of the last compound in the given window and the retention time of the first compound in the next window. Initially, the retention times of the compounds are determined from a scan analysis of a mid- or high-level GC/MS calibration standard. The time windows are adjusted as necessary when the GC column or the guard column are changed or trimmed.

Dwell times for the SIM windows in Table 6 are set to achieve 10–20 scans per peak.

For SIM analyses using Table 7, the start times for windows are determined using formulae such as those shown. These formulae are determined from the analysis of an appropriate NIST SRM or a project-specific sample of oil that has been diluted with dichloromethane then cleaned up by a silica/alumina column and size-exclusion HPLC as described above for tissue samples. The SRM or oil sample is analyzed by GC/MS using scan

Table 6. SIM windows for quantitating selected PAHs in tissue and water samples.

Approximate time window (minutes)	Compounds	Quantitation ion,* confirmation ion* (daltons)
1) 10 to 12	Naphthalene-d8	136
	Naphthalene	128, 127
	2-methylnaphthalene	142, 141
	1-methylnaphthalene	142, 141
2) 12 to 13	2,6-dimethylnaphthalene	156, 141
3) 13 to 14	Hexamethylbenzene	147
	Acenaphthylene	152, 151
	Acenaphthene-d10	164
	Acenaphthene	154, 153
4) 14 to 17	2,3,5-trimethylnaphthalene	170, 155
	Fluorene	166, 165
5) 17 to 20	Dibenzothiophene	184, 139
	Phenanthrene-d10	188
	Phenanthrene	178, 176
	Anthracene	178, 176
6) 20 to 29	3-methylphenanthrene	192, 191
	1-methylphenanthrene	192, 191
	1,7-dimethylphenanthrene	192, 191
	Fluoranthene	202, 101
	Pyrene	202, 101
7) 29 to 42	Benz[*a*]anthracene	228, 226
	Chrysene + triphenylene	228, 226
	Benzo[*b*]fluoranthene	252, 250
	Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene	252, 250
	Benzo[*e*]pyrene	252, 250
	Benzo[*a*]pyrene-d12	264
	Benzo[*a*]pyrene	252, 250
	Perylene	252, 250
8) 42 to 53	Indeno[1,2,3-*cd*]pyrene	276, 138
	Dibenz[*a,h*]anthracene + dibenz[*a,c*]anthracene	278, 139
	Benzo[*ghi*]perylene	276, 138

* Nominal mass.

Table 7. SIM windows for quantitating PAHs, including sums of alkyl isomers in tissue and water samples.

Approximate time window (minutes)	Compounds	Quantitation ion,* confirmation ion* (daltons)
1) 10 to 13.5	Naphthalene-d8	136
	Naphthalene	128, 127
	C1-naphthalenes	142, 141
	C2-naphthalenes	156, 141
2) 13.5 to 14.8 Start = 2,6-dimethylnaphthalene retention time (RT) plus 0.2 min.	C2-naphthalenes	156, 141
	Hexamethylbenzene	147
	Acenaphthylene	152, 151
	Acenaphthene-d10	164
	Acenaphthene	154, 153
	C3-naphthalenes	170, 155
3) 14.8 to 17 Start = 2,3,5-trimethylnaphthalene RT minus 0.75 min	C3-naphthalenes	170, 155
	C4-naphthalenes	184, 169
	Fluorene	166, 165
4) 17 to 20 Start = Dibenzothiophene RT minus 1.7 min	C4-naphthalenes	184, 169
	C1-fluorenes	180, 179
	C2-fluorenes	194, 193
	C3-fluorenes	208, 193
	Dibenzothiophene	184, 139
	Phenanthrene-d10	188
	Phenanthrene	178, 176
	Anthracene	178, 176
5) 20 to 23 Start = Phenanthrene RT plus 0.9 min	C2-fluorenes	194, 193
	C3-fluorenes	208, 193
	C1-dibenzothiophenes	198, 197
	C1-phenanthrenes/anthracenes	192, 191
	C2-dibenzothiophenes	212, 211
	C2-phenanthrenes/anthracenes	206, 191
6) 23 to 25 Start = Fluoranthene RT minus 1.1 min	Fluoranthene	202, 101
	C3-fluorenes	208, 193
	C2-phenanthrenes/anthracenes	206, 191
	C3-phenanthrenes/anthracenes	220, 205
	C2-dibenzothiophenes	212, 211
	C3-dibenzothiophenes	226, 211
	C4-dibenzothiophenes	240, 225

Table 7 continued. SIM windows for quantitating PAHs, including sums of alkyl isomers in tissue and water samples.

Approximate time window (minutes)	Compounds	Quantitation ion,* confirmation ion* (daltons)
7) 25 to 28	Pyrene	202, 101
Start = Pyrene RT minus	Retene	234, 219
0.2 min	C3-dibenzothiophenes	226, 211
	C4-dibenzothiophenes	240, 225
	C3-phenanthrenes/anthracenes	220, 205
	C4-phenanthrenes/anthracenes	234, 219
	C1-fluoranthenes/pyrenes	216, 215
8) 28 to 32	Benz[*a*]anthracene	228, 226
Start = Pyrene RT plus	Chrysene + triphenylene	228, 226
2.85 min	C4-dibenzothiophenes	240, 225
	C4-phenanthrenes/anthracenes	234, 219
	C1-fluoranthenes/pyrenes	216, 215
	C2-fluoranthenes/pyrenes	230, 215
	C3-fluoranthenes/pyrenes	244, 229
9) 32 to 36	C3-fluoranthenes/pyrenes	244, 229
Start = Chrysene RT plus	C4-fluoranthenes/pyrenes	258, 243
0.3 min	C1-benz[*a*]anthracenes/chrysenes	242, 241
	C2-benz[*a*]anthracenes/chrysenes	256, 241
10) 36 to 44	C2-benz[*a*]anthracenes/chrysenes	256, 241
Start =	C3-benz[*a*]anthracenes/chrysenes	270, 255
Benzo[*b*]fluoranthene RT	C4-benz[*a*]anthracenes/chrysenes	284, 269
minus 0.6 min	Benzo[*b*]fluoranthene	252, 250
	Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene	252, 250
	Benzo[*e*]pyrene	252, 250
	Benzo[*a*]pyrene-d12	264
	Benzo[*a*]pyrene	252, 250
	Perylene	252, 250
11) 44 to 53	Indeno[1,2,3-*cd*]pyrene	276, 138
Start = Indeno[1,2,3-*cd*]-	Dibenz[*a,h*]anthracene + dibenz[*a,c*]anthracene	278, 139
pyrene RT minus 0.8 min	Benzo[*ghi*]perylene	276, 138

* Nominal mass.

mode, scanning from 60 to 300 daltons, to identify the groups of alkyl PAH isomers (Table 2) and their retention times in the sample, as well as the retention times of the individual PAH analytes that are not present in the calibration standards. The windows are set such that multiple groups of isomers are monitored in more than one SIM window. This is because, in many cases, the elution of one or more groups of isomers overlap (e.g., the elution of C2-phenanthrenes/ anthracenes partially overlaps with that of C3-fluorenes and C3-phenanthrenes/anthracenes), and the signals are optimized by minimizing and balancing the number of ions per window. The formulae shown in Table 7 are derived for the GC column in use to relate the start times of SIM windows to retention times of PAHs that are in the calibration standard. The resulting formulae can be used to update the SIM-window start times when retention times shift, for example, after the GC column or the guard column are changed or trimmed.

Dwell times for the SIM windows in Table 7 are set at 65 for all ions in windows 1 through 10 for consistent response, and 100 for all ions in window 11 for smoother signals.

The quantitation ions for PAHs in tissues and water shown in Table 6 and Table 7 are also the ions used for quantitating PAHs in sediments.

5.2.2. GC/MS SIM Windows for PAHs, Alkyl PAH Isomers, and Alkanes

If water samples are to be analyzed for the alkanes (pristane, phytane, n-heptadecane, and n-octadecane), the alkanes are quantitated concurrently with the PAHs using the SIM windows in Table 7, modified to include the quantitation ion 71 and the confirmation ion 57 in window 5 for pristane and n-heptadecane and in window 6 for phytane and n-octadecane, as needed.

5.2.3. Verification of GC/MS Performance for PAHs and Alkyl PAH Isomers

The sensitivity of the GC/MS is checked by analyzing the lowest level of PAH GC calibration standard that will be used to quantitate PAHs in the samples (Level 1 for tissues and water, Level 3 for sediments).

5.2.4. GC/MS Analysis of Sediment Samples for PAHs

One or more batches of sediment samples are analyzed in a sequence that includes one each of PAH GC calibration standard Levels 3, 4, 5, 6, 8, and 9, alternating with the samples, plus one Level 7 before the first sample and replicate Level 7 analyses at the middle and end of each batch. Included in this sequence is a representative sediment sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

5.2.5. GC/MS Analysis of Tissue Samples for PAHs and Alkyl PAH Isomers

One or more batches of tissues samples are analyzed in a sequence that includes one each of PAH GC calibration standard Levels 1, 2, 3, 5, and 6, alternating with the samples in the sequence, plus one Level 4 before the first sample and replicate Level 4 analyses at the middle and end of each batch. Included in this sequence is one injection of a representative tissue sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

5.2.6. GC/MS Analysis of Water Samples for PAHs, Alkyl PAH Isomers, and Alkanes

One or more batches of water samples are analyzed in a sequence that includes one each of PAH plus alkanes GC calibration standard Levels 1, 2, 4, 5, and 6, alternating with the samples in the sequence, plus one Level 3 before the first sample and replicate Level 3 analyses at the middle and end of each batch. Included in this sequence is one injection of a representative water sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

5.3. GC/MS for Quantitating POPs

The GC oven temperature is programmed for POPs as follows.

Initial temperature:	1 min at 80°C
First rate:	10°C/min to 150°C
Isothermal hold:	0 min
Second rate:	0.5°C/min to 195°C
Isothermal hold:	0 min
Third rate:	3°C/min to 315°C
Isothermal hold:	15 min at 315°C
Total run time:	153 min

5.3.1. GC/MS SIM Windows for POPs

Sediment, tissue, and water samples are analyzed using SIM mode, scanning only the quantitation ions during the specified time window as shown in Table 8. For SIM analyses using Table 8, the actual time for ending a given window and starting the next window is determined as approximately the midpoint between the retention time of the last compound in the given window and the retention time of the first compound in the next window. Initially, the retention times of the compounds are determined from a scan analysis of a mid-level or high-level GC/MS calibration standard. The time windows are adjusted as necessary when the GC column is changed or the guard column is trimmed.

Dwell times for the SIM windows in Table 8 are set to achieve 10–20 scans per peak.

5.3.2. Verification of GC/MS Performance for POPs

The sensitivity of the GC/MS is checked by analyzing the lowest level of POP GC calibration standard that will be used to quantitate POPs in the samples.

5.3.3. GC/MS Analysis of Sediment Samples for POPs

One or more batches of sediment samples are analyzed in a sequence that includes one each of POP GC calibration standard Levels 2 through 5 and Level 7 alternating with the samples, plus one Level 6 before the first sample and replicate Level 6 analyses at the middle and end of each batch. Included in this sequence is one injection of a representative sediment sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

Table 8. SIM windows for quantitating POPs in tissue, sediment, and water samples.

Approximate time window (minutes)	Compounds	Quantitation ions[a, b]
1) 20 to 28	Tetrachloro-*o*-xylene	207
	Tetrachloro-*m*-xylene	207
2) 28 to 35	Dibromooctafluorobiphenyl	456
	alpha-Hexachlorocyclohexane	181
	Hexachlorobenzene	284
3) 35 to 39	beta-Hexachlorocyclohexane	219
	gamma-Hexachlorocyclohexane	181
4) 39 to 44	PCB 18	256
	PCB 17	256
5) 44 to 54	PCB 31	256
	PCB 28	256
	PCB 33	256
	Heptachlor	272
6) 54 to 64	PCB 52	292
	PCB 49	292
	Aldrin	263
	PCB 44	292
7) 64 to 68	PCB 103	326
8) 68 to 74	Heptachlor epoxide	353
	Oxychlordane	115
	PCB 74	292
	PCB 70	292
	PCB 66	292
	PCB 95	326
9) 74 to 77	*trans*-Chlordane	373
10) 77 to 84	2,4'-DDE	246
	Endosulfan I	241
	PCB 101 + PCB 90	326
	Nonachlor III	409
	cis-Chlordane	373
	PCB 99	326
	trans-Nonachlor	409

Table 8 continued. SIM windows for quantitating POPs in tissue, sediment, and water samples.

Approximate time window (minutes)	Compounds	Quantitation ions[a, b]
11) 84 to 91	Dieldrin	263
	PCB 87	326
	4,4'-DDE	246
	PCB 110	326
	2,4'-DDD	235
12) 91 to 96	PCB 82	326
	PCB 151	360
13) 96 to 99	PCB 149	360
	PBDE 28	405.8, 407.8
	PCB 118	326
14) 99 to 102	*cis*-Nonachlor	409
	4,4'-DDD	235
	2,4'-DDT	235
15) 102 to 106	PCB 153 + PCB 132	360
	PCB 105	326
16) 106 to 109	4,4'-DDT	235
	PCB 138 + PCB 163 + PCB 164	360
	PCB 158	360
17) 109 to 112	PCB 187 + PCB 159 + PCB 182	394
	PCB 183	394
	PCB 128	360
18) 112 to 115	PCB 177	394
	PCB 171	394
	PBDE 49	483.7, 485.7
	PCB 156	360
19) 115 to 120	PCB 180	394
	PBDE 47	483.7, 485.7
	PCB 191	394
	PBDE 66	483.7, 485.7
	Mirex	272
	PCB 170	394
	PCB 199	430

Table 8 continued. SIM windows for quantitating POPs in tissue, sediment, and water samples.

Approximate time window (minutes)	Compounds	Quantitation ions[a, b]
20) 120 to 125	PCB 208	464
	PCB 195	430
	PBDE 100	563.6, 565.6
	PCB 194	430
	PCB 205	430
	PBDE 99	563.6, 565.6
21) 125 to 134	PCB 206	464
	PCB 209	498
	PBDE 85	563.6, 565.6
	PBDE 155	641.5, 643.5
	PBDE 154	641.5, 643.5
	PBDE 153	641.5, 643.5
22) 134 to 142	PBDE 183	561.6, 563.6

[a] Nominal mass, except the exact mass to one significant figure for PBDEs.
[b] For quantitating PBDEs, the signals of two ions are summed.

5.3.4. GC/MS Analysis of Low-lipid Tissue Samples or Water Samples for POPs

One or more batches of low-lipid tissue samples or water samples are analyzed in a sequence that includes one each of POP GC calibration standard Levels 1 through 5 and Level 7 alternating with the samples (Level 1 is optional, depending on the project), plus one Level 6 before the first sample and replicate Level 6 analyses at the middle and end of each batch. Included in this sequence is one injection of a representative tissue or water sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

5.3.5. GC/MS Analysis of High-lipid Tissue Samples for POPs

One or more batches of high-lipid tissue samples are analyzed in a sequence that includes one each of POP GC calibration standard Levels 1 through 5 and Levels 7 through 11 alternating with the samples (Level 1 is optional, depending on the project), plus one Level 6 before the first sample and replicate Level 6 analyses at the middle and end of each batch. Included in this sequence is one injection of a representative tissue sample at the beginning of the sequence, which serves as a GC/MS system conditioner.

6. Calculations of GC/MS Results

6.1. Calculating Analyte Concentrations

The acquired GC/MS data for the samples and calibration standards are processed using the Agilent ChemStation software to determine areas for the analytes' peaks and the internal standards' peaks in the chromatograms. The identifications and integrations of the peaks are performed by the ChemStation software to generate the peak areas, and then they are checked for appropriateness and manually reintegrated as needed.

For quantitating the groups of alkyl PAH isomers (Table 2), the responses for all isomers within a group are summed by integrating the area for all peaks in the entire group to get one response area, and the isomer group is treated as a single analyte. The retention times and relative peak abundances of the isomers determined from a SIM analysis of an appropriate SRM or a project-specific oil sample can be used for guidance. For example, panel A of Figure 4 shows the group of C3-naphthalenes in an oil sample (between arrows) and panel B and panel C show the correspondingly integrated C3-naphthalenes in a brown shrimp sample and a sample of NIST SRM 1974b, respectively. Similarly, the panels of Figure 5 show the chromatograms for C2-fluorenes.

The analyte areas produced by the ChemStation software are then used by a BASIC program written in-house to compute the analyte amounts in the samples (ng analyte per sample) based on point-to-point calibration (equations provided below). The point-to-point calibration method is equivalent to plotting the relative response factors (RRFs) of the calibration standards versus their analyte areas to achieve two-dimensional points. Linear equations generated between each pair of consecutive points are used for the calibration in order to gain a better fit over the entire range of calibration standards than would be provided by a single regression equation, such as linear, weighted linear, or quadratic regression.

For each level of calibration standard (CS), the RRF is computed for each analyte relative to its surrogate standard (SS) as

$$\text{CS RRF} = \frac{\text{CS Analyte Concentration}}{\text{CS Analyte Area}} \times \frac{\text{CS SS Area}}{\text{CS SS Concentration}} \quad (3)$$

For the PAHs listed in Table 1, the surrogate standards (SSs) are naphthalene-d8 for the analytes naphthalene through 1-methylnaphthalene, acenaphthene-d10 for analytes acenaphthene through pyrene, and benzo[*a*]pyrene-d12 for analytes benz[*a*]anthracene through benzo[*ghi*]perylene. For the PAHs including alkyl PAH isomers listed in Table 2, the SSs are naphthalene-d8 for the analytes naphthalene and C1-naphthalenes, acenaphthene-d10 for analytes C2-naphthalenes through C4-fluoranthenes and pyrenes, and benzo[*a*]pyrene-d12 for analytes benz[*a*]anthracene through benzo[*ghi*]perylene. The POP SS is PCB 103 for all

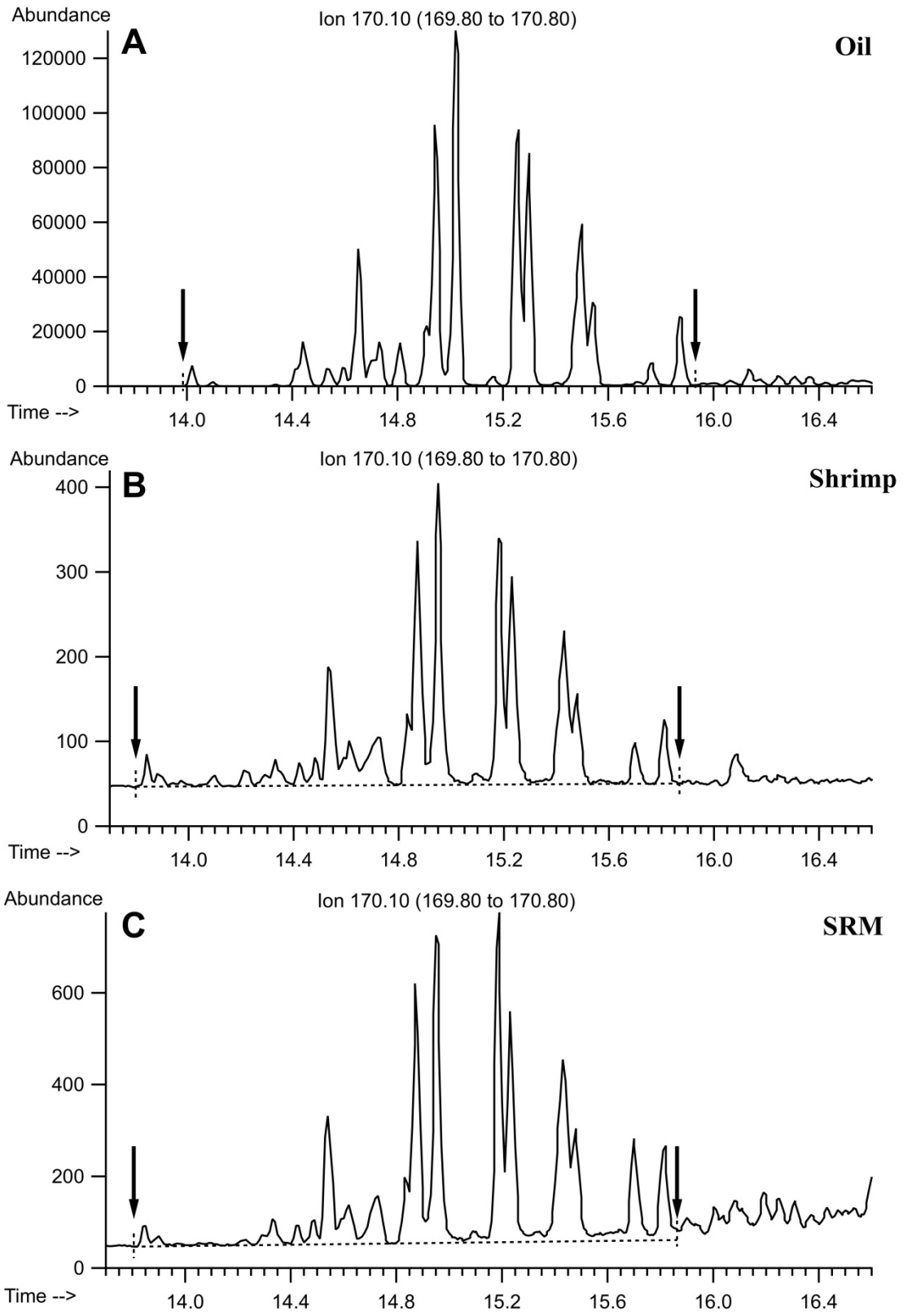

Figure 4. GC/MS SIM chromatograms of C3-naphthalenes in an oil sample (panel A), a brown shrimp sample (panel B), and a sample of NIST SRM 1974b (panel C). Arrows point to the start and end of the integration baseline.

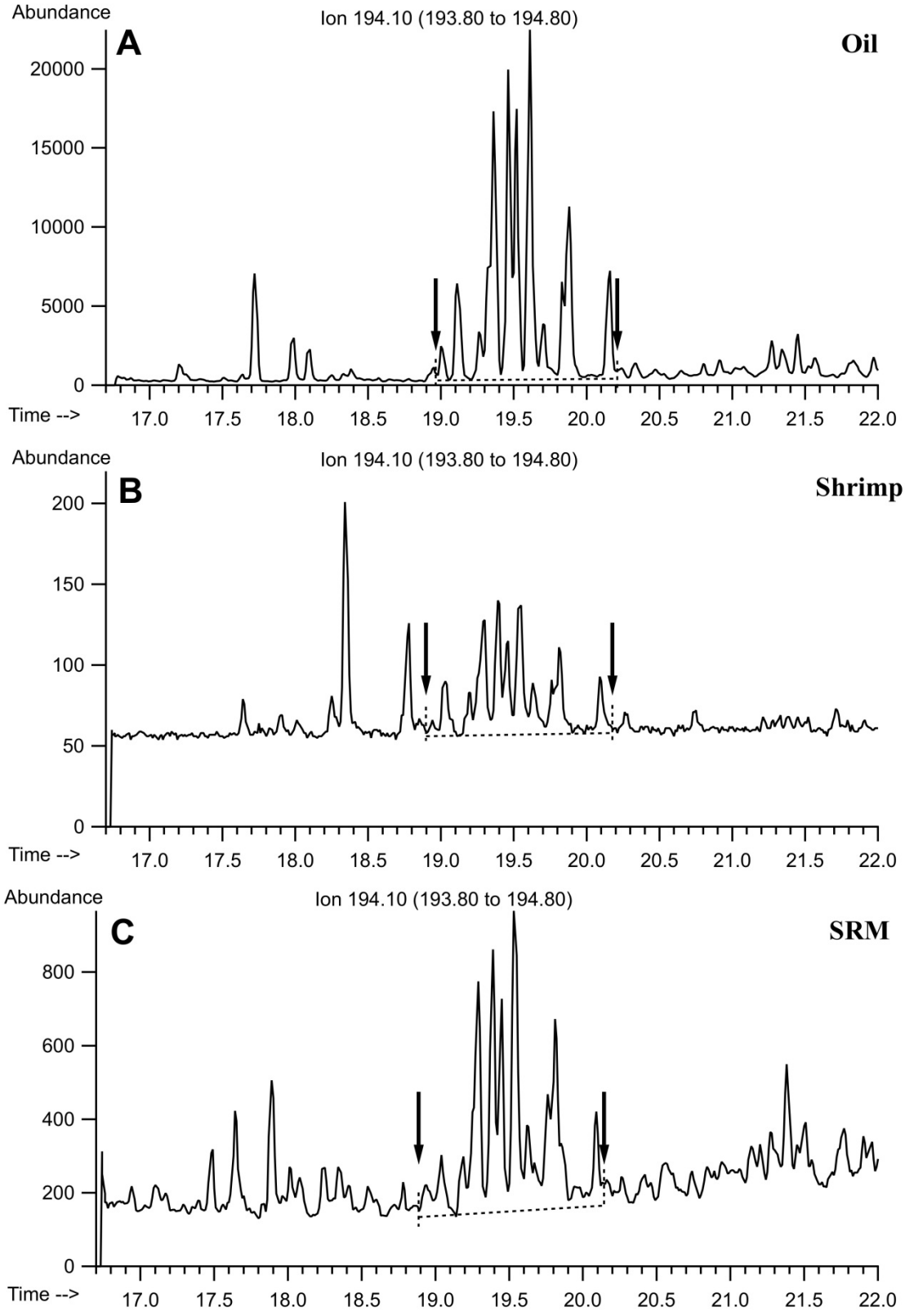

Figure 5. GC/MS SIM chromatograms of C2-fluorenes in an oil sample (panel A), a brown shrimp sample (panel B), and a sample of NIST SRM 1974b (panel C). Arrows point to the start and end of the integration baseline.

analytes in Table 3, except DOB is used as the POP SS for all analytes in cases when endogenous PCB 103 is evident, such as in highly contaminated marine mammal blubber. For the alkanes (pristane, phytane, n-heptadecane, and n-octadecane), the SS is acenaphthene-d10.

For the analytes not contained in the CSs, the response factor of a similar analyte is used, that is, 1-methylphenanthrene for retene, chrysene for chrysene + triphenylene, benzo[*k*]fluoranthene for benzo[*k*]fluoranthene + benzo[*j*]fluoranthene, dibenz[*a*,*h*]anthracene for dibenz[*a*,*h*]anthracene + dibenz[*a*,*c*]anthracene, PCB 101 for PCB 101 + PCB 90, PCB 138 for PCB 138 + PCB 163 + PCB 164, PCB 153 for PCB 153 + PCB 132, PCB 187 for PCB 187 + PCB 159 + PCB 182, *trans*-nonachlor for nonachlor-III, and as shown in Table 9 for the groups of alkyl PAH isomers.

Next, specific for each sample, a sample RRF for each analyte relative to its surrogate standard is computed by interpolating between the RRFs of the two consecutive calibration standards whose areas for that analyte bracket the area of the analyte in the sample, as

$$\text{Sample RRF} = \text{Lower CS RRF} + \frac{\text{Sample Analyte Area} - \text{Lower CS Analyte Area}}{\text{Upper CS Analyte Area} - \text{Lower CS Analyte Area}} \times (\text{Upper CS RRF} - \text{Lower CS RRF}) \quad (4)$$

Table 9. Compounds used for response factors to quantitate isomer groups of alkyl PAH isomers.

Analyte	**Compound used for response factor**
C2-Naphthalenes	2,6-Dimethylnaphthalene
C3-Naphthalenes	2,3,5-Trimethylnaphthalene
C4-Naphthalenes	2,3,5-Trimethylnaphthalene
C1-Fluorenes	Fluorene
C2-Fluorenes	Fluorene
C3-Fluorenes	Fluorene
C1-Dibenzothiophenes	Dibenzothiophene
C2-Dibenzothiophenes	Dibenzothiophene
C3-Dibenzothiophenes	Dibenzothiophene
C4-Dibenzothiophenes	Dibenzothiophene
C1-Phenanthrenes and anthracenes	1-Methylphenanthrene
C2-Phenanthrenes and anthracenes	1,7-Dimethylphenanthrene
C3-Phenanthrenes and anthracenes	1,7-Dimethylphenanthrene
C4-Phenanthrenes and anthracenes	1,7-Dimethylphenanthrene
C1-Fluoranthenes and pyrenes	Fluoranthene
C2-Fluoranthenes and pyrenes	Fluoranthene
C3-Fluoranthenes and pyrenes	Fluoranthene
C4-Fluoranthenes and pyrenes	Fluoranthene
C1-Benz[*a*]anthracenes and chrysenes	Chrysene
C2-Benz[*a*]anthracenes and chrysenes	Chrysene
C3-Benz[*a*]anthracenes and chrysenes	Chrysene
C4-Benz[*a*]anthracenes and chrysenes	Chrysene

where Lower CS is the calibration standard whose analyte area is closest to and smaller than that of the sample's analyte area and Upper CS is the calibration standard whose analyte area is closest to and larger than that of the sample's analyte area.

The amount of each analyte in each sample (ng Analyte per Sample) is then calculated as

$$\text{ng Analyte/Sample} = \text{Sample RRF} \times \text{Sample Analyte Area} \times \frac{\text{Sample SS ng}}{\text{Sample SS Area}} \quad (5)$$

where Sample SS ng is the ng amount of surrogate standard added to the sample prior to extraction.

These analyte amounts for the samples are then used by a Filemaker Pro database to compute the analyte concentrations in the samples. Concentrations on a wet weight basis or a dry weight basis are calculated as

$$\text{ng/g wet weight} = \frac{\text{ng Analyte per Sample}}{\text{Sample Weight (g)}} \quad (6)$$

and

$$\text{ng/g dry weight} = \frac{\text{ng Analyte per Sample} \times 100\%}{\text{Sample Weight (g)} \times \text{\% Dry Weight}} \quad (7)$$

where the % Dry Weight is determined as described in Subsection 8.1.

6.2. Calculating Internal Standard Recoveries

The percent recoveries of the PAH SSs (naphthalene-d8, acenaphthene-d10, and benzo[*a*]pyrene-d12) are computed for each sample, including the PAH Standard Check Solution, based on the HPLC IS (phenanthrene-d10). Furthermore, the PAH HPLC IS recovery is computed based on the PAH GC IS (HMB). For these calculations, a single analysis of the Level 3 GC CS in the case of SIM analyses, or the Level 6 GC CS in the case of scan analyses, is used for the RRFs of the SSs.

The percent recovery of the POP SS (PCB 103) is computed for each sample, including the POP Standard Check Solution, based on the POP HPLC IS (TCMX). Furthermore, the POP HPLC IS recovery is computed based on the POP GC IS (TCOX). For these calculations, a single analysis of the Level 6 GC CS is used for the RRF of the SS.

The percent recovery of each surrogate standard (SS % Recovery) is calculated as

$$\text{SS \% Recovery} = \text{CS SS RRF} \times \frac{\text{Sample SS Area}}{\text{Sample SS ng}} \times \frac{\text{Sample HPLC IS ng} \times 100\%}{\text{Sample HPLC IS Area}} \quad (8)$$

where

$$\text{CS SS RRF} = \frac{\text{CS SS Concentration}}{\text{CS SS Area}} \times \frac{\text{CS HPLC IS Area}}{\text{CS HPLC IS Concentration}} \quad (9)$$

and Sample HPLC IS ng is the ng amount of HPLC internal standard added to the sample.

The percent recovery of the HPLC internal standard (HPLC IS % Recovery) is monitored for in-house QA information and is calculated as

$$\text{HPLC IS \% Recovery} = \text{CS HPLC IS RRF} \times \frac{\text{Sample HPLC IS area}}{\text{Sample HPLC IS ng}} \times \frac{\text{Sample GC IS ng} \times 100\%}{\text{Sample GC IS Area}} \quad (10)$$

where

$$\text{CS HPLC IS RRF} = \frac{\text{CS HPLC IS Concentration}}{\text{CS HPLC IS Area}} \times \frac{\text{CS GC IS Area}}{\text{CS GC IS Concentration}} \quad (11)$$

and Sample GC IS ng is the ng amount of GC internal standard added to the sample.

6.3. Calculating and Reporting Lower Limits of Quantitation

The LOQ for a given analyte in a given sample is the concentration that would be calculated if the analyte had a GC/MS response area equivalent to that analyte's area in the lowest level CS used in the calibration for that analyte (not all levels are used for some analytes). When an analyte is not detected in a sample or has an area that is smaller than its area in the lowest level CS used, the concentration of the analyte in that sample is reported to be less than the value of its LOQ.

6.4. Calculating and Reporting Estimated Concentrations for Analytes Exceeding the Calibration Range

When an analyte in a given sample has a GC/MS area that is larger than its area in the highest level CS used in the calibration, that sample is diluted as needed (up to fivefold) to get the analyte area within the calibration range then reanalyzed by GC/MS along with its corresponding QA samples. Alternatively, the analyte amount is calculated using the RRF of that analyte in the highest level CS used for that analyte (not all levels are used for some analytes), and the concentration is footnoted as exceeding the calibration range and is therefore an estimate.

7. Quality Assurance for PAHs, POPs, and Alkanes

QA measures are incorporated into the analyses of each batch of samples according to the Environmental Chemistry Program Quality Assurance Plan. Specific QA goals for batches and individual samples, as well as the percent of the total results that must meet the goals, depend on the project for which the samples are analyzed and the purpose of the data. Typical goals are presented here. When QA goals are not met, the data may be qualified or the sample(s) reanalyzed.

7.1. GC/MS Stability

The stability of the GC/MS is evaluated using the repetitions of the mid-level GC CS analyzed intermittently in the sequence of samples and other GC calibration standards. The GC/MS is considered stable if, for each analyte, the percent relative standard deviation (RSD) of the analyte responses relative to its SS responses is ≤15%.

7.2. Surrogate and Internal Standard Recoveries

The percent recoveries of SSs in all samples are considered acceptable if they are between 60% and 130%. The percent recoveries of these standards in the Standard Check Solutions are monitored for problems with the SS and IS solutions. The percent recoveries of HPLC ISs are monitored in-house and do not have QA criteria.

7.3. Analyte Concentrations in Reference Materials

At least one sample of an appropriate NIST SRM is analyzed in each batch of tissue or sediment samples to indicate the accuracy of the data for the entire batch. An appropriate NIST SRM for water analyses is not available at this time. NIST provides certified concentrations and uncertainty values for many of the individual compounds in Table 1, Table 2, and Table 3. For the analytes having NIST certified concentrations, upper and lower control limits are set as is done by NIST in the Intercomparison Exercise Program for Organics in the Marine Environment, and are defined as

$$\text{Upper Control Limit} = 1.3 \times (\text{Certified Concentration} + \text{Uncertainty Value}) \quad (12)$$

and

$$\text{Lower Control Limit} = 0.7 \times (\text{Certified Concentration} - \text{Uncertainty Value}) \quad (13)$$

The accuracy of the SRM result is considered acceptable if the measured concentration falls within the Upper and Lower Control Limits. This goal does not apply to analytes with concentrations below their LOQ when their LOQ is greater than their Lower Control Limit. The

analysis of the SRM is acceptable if at least 70% of the analytes having NIST certified concentrations are within their control limits. Analytes which do not have NIST certified concentrations do not have QA goals for accuracy; however, their SRM results can be monitored and compared to NIST reference concentrations, where available, or to previous in-house results.

7.4. Precision of Replicate Samples

When three or more replicate samples are analyzed, the precision of the results can be evaluated based on the RSD of the concentrations of each analyte. The precision is considered acceptable if the RSD is ≤15% for at least 90% of the analytes with concentrations greater than 1 ng/g. For duplicate samples, this translates to a relative percent difference of ≤30% for at least 90% of the analytes.

7.5. Method Blank Contamination

A method blank is analyzed with each batch of samples to monitor for contamination from laboratory sources. Ideally, analytes would not be detected in the method blank above their LOQ. However, if analytes are measured in the method blank, their concentrations need to be compared to the concentrations in the sediment, tissue, or water samples in the same batch. For each project, criteria need to be determined for contaminants in the method blank; for example, the method blank contamination might be considered acceptable if no more than 10% of the analytes have concentrations exceeding two times the LOQ. Also, analytes in tissue, sediment, or water samples having concentrations between the LOQ and four times the concentration in the method blank for the batch might be footnoted or qualified as such. Analyte concentrations in method blanks are not subtracted from analyte concentrations in field or SRM samples.

The HPLC blank is monitored in-house and does not have QA criteria.

7.6. Spiked Blank Recoveries

At least one spiked blank water sample is analyzed in each batch of water samples to indicate the accuracy of the data for the entire batch, because an appropriate NIST SRM for water analyses is not available at this time. The percent recoveries of spikes in spiked blank samples are considered acceptable if they are between 60% and 130%. The percent recoveries of spikes in the Standard Check Solutions are monitored for problems with the spike solutions.

8. Gravimetric Determinations

8.1. Dry Weight Determination

The sample number is etched on the tab of a 43-mm id aluminum weighing pan.

The pan is placed in a drying oven at 120°C overnight then cooled in a desiccator for 30 minutes.

The pan is weighed to the nearest 0.001 g, and the weight is recorded as the "Pan Weight."

For sediment samples, using a spatula, the sediment is stirred in its container until thoroughly homogenized. Standing water was decanted from the sediment sample container and all pebbles, shells, biota, and other extraneous material were discarded previously in Section 3; no further decanting of excess water is done here so that the water content is the same as when the portion of sediment was taken for PAH and POP analysis.

The sediment (2 g, ±0.5) is placed into the pan, and the pan is weighed to the nearest 0.001 g. The weight is recorded as the "Pan w/Wet Sample Weight."

For tissue samples, using a spatula, the tissue is stirred in its container to ensure that it is homogeneous. The tissue was previously homogenized in Section 3.

The tissue (1 g, ±0.5) is placed into the pan, and the pan is weighed to the nearest 0.001 g. The weight is recorded as the "Pan w/Wet Sample Weight."

For sediment or tissue samples, the pan is placed in a drying oven at 120°C for 24 hours then cooled in a desiccator for 30 minutes.

The pan is weighed to the nearest 0.001 g, and the weight is recorded as the "Pan w/Dry Sample Weight."

The percent dry weight of the sample is determined as

$$\% \text{ Dry Weight} = \frac{(\text{Pan w/Dry Sample Weight} - \text{Pan Weight}) \times 100\%}{\text{Pan w/Wet Sample Weight} - \text{Pan Weight}} \quad (14)$$

The % Dry Weight values for QA samples are monitored in-house and do not have QA criteria.

8.2. Total Extractables Determination

The pan containing the sample for total extractables from Section 3 is placed on a covered rack in the hood, and the solvent is allowed to completely evaporate (approximately 1–2 hours).

The pan is dried in a 50°C oven for 2 hours then cooled in a desiccator overnight.

8.2.1. Batches of Tissue Samples Requiring Macro Total Extractables Determination

The pan is weighed to the nearest 0.001g, and the weight is recorded as the "Pan w/TE Weight."

The percent total extractables (% TE) content of the sample is calculated as

$$\%\ TE = \frac{(\text{Pan w/TE} - \text{Pan Weight}) \times (\text{ASE Vial w/Extract Weight} - \text{ASE Vial Weight}) \times 100\%}{(\text{ASE Vial w/Extract Weight} - \text{ASE Vial w/out TE Extract Weight}) \times \text{Sample Weight}} \quad (15)$$

8.2.2. Batches of Tissue Samples Requiring Micro Total Extractables Determination

The pan is weighed to the nearest 0.001mg, and the weight is recorded as the "Pan w/TE Weight."

The percent total extractables (% TE) content of the sample is calculated as

$$\%\ TE = \frac{(\text{Pan w/TE} - \text{Pan Weight}) \times (\text{ASE Vial w/Extract Weight} - \text{ASE Vial Weight}) \times 100\%}{(\text{GC Vial w/TE Extract Weight} - \text{GC Vial Weight}) \times \text{Sample Weight}} \quad (16)$$

The % TE values for QA samples are monitored in-house and do not have QA criteria.

9. Lipid Classes

Aliquots of sample extracts are analyzed for percentages of lipid classes by TLC/FID. Lipid classes are separated by TLC and detected by FID, producing a chromatogram for each analysis, where the detector's response to the compounds produces peaks. The area under each peak is proportional to the concentration of the lipid class in the sample or standard analyzed.

The portion of sample extract in the 1.5-mL vial for lipid classes from Section 3 is stored at between 4 and −20°C until analyzed.

For low-lipid tissues (<10% lipid), the lipid classes extract portion is reduced in volume to between 10 and 100 µL using a gentle stream of ultra-pure nitrogen, and the cap is immediately replaced. The weight of the concentrated extract in the capped vial is weighed to the nearest 0.001 g, and the weight is recorded as "Iatro Vial w/Conc Extract Weight."

9.1. Separation of Lipid Classes

A TLC development tank containing 70 mL of hexane/diethyl ether/formic acid [60:10:0.02 (v:v:v)] and a 5.75″ × 7.35″ piece of filter paper is set in a fume hood (temperature range 20–25°C). To ensure that the development tank is completely saturated, it is then allowed to sit at least 30 minutes prior to placing the first set of Chromarods in the tank.

Ten Chromarods are placed into a SD-5 Chromarod holder, and each rod is blank scanned (from top to bottom at a speed of 60 seconds per scan) one to three times by the Iatroscan to remove any organic compounds that are present and "activate" the Chromarod.

The rod holder containing the Chromarods is removed from the Iatroscan and placed onto a spotting guide.

The Chromarods are spotted with one sample extract or one calibration standard per rod as follows. A 1-µL aliquot is carefully spotted onto a Chromarod near the base of the Chromarod using a 2-µL syringe, keeping the spot as small as possible in order to provide good separation of the lipid classes and reproducible analyses. Each sample extract is analyzed in duplicate. Also, within each holder of 10 Chromarods, one Chromarod is spotted with a continuing calibration check standard (one of CSs Levels 1–5) to be analyzed concurrently with sample extracts.

The rod holder containing the Chromarods is placed in the TLC development tank for exactly 24 minutes to separate the various lipid classes listed in Section 1 (wax esters/sterol esters, triglycerides, free fatty acids, cholesterol, and phospholipids/other polar lipids). Then the rod holder is removed immediately from the tank, and the remaining solvent is allowed to evaporate for 2 minutes in a 60°C oven.

The developed Chromarods in the holder proceed to TLC/FID analysis.

9.2. TLC/FID Analysis

The operating conditions for the Iatroscan FID are as follows.

Hydrogen flow rate:	160 mL/min
Air flow rate:	2,000 mL/min
Scan rate:	30 s/scan

The Chromarod holder containing the 10 Chromarods is placed in the Iatroscan FID.

Each Chromarod is sequentially passed through the hydrogen flame to ionize the sample extract or standard, and the detected changes in current are recorded by the Empower data acquisition system to generate the chromatogram.

The TLC/FID calibration standards are analyzed at least every 4 weeks to generate the calibration equations for each lipid class as described below. As noted above, a continuing calibration check standard (one of CSs Levels 1–5) is analyzed concurrently with the sample extracts within each holder of 10 Chromarods.

9.3. Calculations of Lipid Class Concentrations

The acquired TLC/FID data for the samples and calibration standards are processed using the Empower software to determine areas for the lipid classes' peaks in the samples' chromatograms and for individual compounds' peaks in the CSs' chromatograms. The identification and integration of the peaks are performed by the Empower software to generate the peak areas, and then they are checked for appropriateness and manually reintegrated as needed.

The individual compounds used to calibrate or quantitate the corresponding lipid classes are as follows.

Lipid class	**Lipid compound used for response factor**
Wax esters and sterol esters	Lauryl stearate
Triglycerides	Triolein
Free fatty acids	Oleic acid
Cholesterol	Cholesterol
Phospholipids and other polar lipids	L-α-phosphatidylcholine

The lipid areas produced by the Empower software are then used by a FileMaker Pro database to compute the amounts of each lipid class in the samples (mg lipid) based on point-to-point calibration (equations provided below). The point-to-point calibration method is equivalent to plotting the response factors (RFs) of the CSs versus their analyte areas to achieve two-dimensional points. Linear equations generated between each pair of consecutive points are used for the calibration in order to gain a better fit over the entire range of CSs than would be provided by a single regression equation, such as linear, weighted linear, or quadratic regression.

For each level of calibration standard (CS), a RF is computed for each lipid as

$$\text{CS RF} = \frac{\text{CS Lipid Concentration (mg/mL)}}{\text{CS Lipid Area}} \quad (17)$$

Next, specific for each sample, a sample RF for each lipid class is computed by interpolating between the RFs of the two consecutive calibration standards whose areas for that lipid bracket the area of the lipid class in the sample, as

$$\text{Sample RF} = \text{Lower CS RF} + \frac{(\text{Sample Lipid Class Area} - \text{Lower CS Lipid Area}) \times (\text{Upper CS RF} - \text{Lower CS RF})}{\text{Upper CS Lipid Area} - \text{Lower CS Lipid Area}} \quad (18)$$

where Lower CS is the calibration standard whose lipid area is closest to and smaller than that of the sample's lipid class area, and Upper CS is the calibration standard whose lipid area is closest to and larger than that of the sample's lipid class area. If the Sample Lipid Class Area is larger than the Level 1 lipid area, then the sample is diluted to get the area within the calibration range and the sample is reanalyzed; if the Sample Lipid Class Area is smaller than the Level 6 lipid area, then the Sample Lipid Class Area is set to zero.

The concentration of each lipid class in each sample is then calculated in terms of mg lipid per g sample wet weight as

$$\frac{\text{mg Lipid Class}}{\text{g Sample}} = \frac{\text{Sample RF} \times \text{Sample Lipid Class Area} \times (\text{ASE Vial w/Extract Weight} - \text{ASE Vial Weight})}{1.33\ \text{g/mL} \times \text{Iatro Extract Concentration Factor} \times \text{Sample Weight}} \quad (19)$$

where 1.33 g/mL is the density of dichloromethane, and if the extract in the Iatro vial is concentrated prior to analysis,

$$\text{Iatro Extract Concentration Factor} = \frac{\text{Iatro Vial w/Extract Weight} - \text{Iatro Vial Weight}}{\text{Iatro Vial w/Conc Extract Weight} - \text{Iatro Vial Weight}} \quad (20)$$

or if the extract in the Iatro vial is not concentrated prior to analysis,

$$\text{Iatro Extract Concentration Factor} = 1 \quad (21)$$

The percent of each lipid class per sum of all lipid classes is calculated as

$$\text{Percent Lipid Class} = \frac{\text{Lipid Class Concentration} \times 100\%}{\text{Sum of All Lipid Classes' Concentrations}} \quad (22)$$

Duplicate TLC/FID analyses are performed for each lipid extract, and the mean value is reported for each lipid class's Percent Lipid Class.

The limits of quantitation for each of the five lipids contained in the calibration standards are the following: 0.63 mg/mL lauryl stearate, 0.63 mg/mL triolein, 0.63 mg/mL oleic acid, 0.13 mg/mL cholesterol, and 0.13 mg/mL L-α-phosphatidylcholine.

9.4. Quality Assurance for Lipid Classes

QA measures are incorporated into the analyses of each batch of samples according to the Environmental Chemistry Program Quality Assurance Plan. Specific QA goals for batches and individual samples, as well as the percent of the total results that must meet the goals, depend on the project for which the samples are analyzed and the purpose of the data. Typical goals are presented here. When QA goals are not met, the data may be qualified or the sample(s) reanalyzed.

9.4.1. TLC/FID Stability

External standard curves for each lipid class are created at least every 4 weeks. To determine whether the Iatroscan TLC/FID is operating properly, a calibration standard will be analyzed with each Chromarod rack that consists of 10 Chromarods. If the concentration of a lipid class in the calibration standard is not within ±20% of the known concentration of that compound, the field samples will be reanalyzed (unless the lipid class is not measured in the associated field samples).

9.4.2. Percent Summed Lipid Value for Reference Materials

To monitor the accuracy of the lipid extraction and analyses, the Percent Summed Lipid value of the SRM determined by the TLC/FID method is compared to the NIST percent nonvolatile extractable material value. The accuracy of the NIST SRM result is considered acceptable if the Percent Summed Lipid value is within 35% of either end of NIST's "95% confidence interval for the mean" for the NIST percent nonvolatile extractable material value. However, for low-lipid containing SRMs (<2%), the TLC/FID value is considered acceptable if the Percent Summed Lipid value is within 50% of either end of NIST's "95% confidence interval for the mean" for the NIST percent nonvolatile extractable material value. There are no NIST certified values for lipid classes.

9.4.3. Precision of Replicate Samples

When three or more replicate samples are extracted and analyzed, the precision of the results can be evaluated based on the RSD of the percentages of each lipid class and the Percent Summed Lipid value. The precision is considered acceptable if the RSD is ≤25% for analytes with concentrations greater than their LOQ. For duplicate samples, this translates to a relative percent difference of ≤50%. This does not apply to the duplicate TLC/FID analyses of the same extract.

9.4.4. Method Blank Contamination

The method blank is analyzed to determine whether the extraction solvents, glassware, or other supplies were free of lipid contaminants. Our acceptance criterion is that no peak is to be detected above the noise level in a method blank.

10. Conclusions

The analytical methods presented here resulted from research, development, and application by chemists in the Environmental Chemistry Program. These methods will continue to be evaluated and upgraded as feasible for improved accuracy, precision, sensitivity, robustness, safety, and cost-effectiveness, as well as for minimizing contamination from laboratory materials and from sample-to-sample carryover. QA measures are included, which are monitored and reported for every batch of samples. Our methods are provided here in detail for complete documentation as well as for ease of use by other laboratories.

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