NOAA Technical Memorandum NMFSF/NWC-64

Standard Analytical Procedures of the NOAA National Analytical Facility, 1984-85

Extractable Toxic Organic Compounds.

Prepared for
The NOAA National Status and Trends Program

By
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October 1984

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
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Numerous recent studies demonstrate associations between organic chemical contamination of the aquatic environment and impacts on environmental health and, potentially, on human health (cf. Malins et al. 1984). If the results of one study are to be compared with those of another, uniform analytical methods for the chemicals will be required. To meet this need, NOAA's National Analytical Facility (NAF) prepared this Technical Memorandum as a methods manual for extractable organic chemicals in marine sediments and tissues. It applies specifically to the organic analytes (i.e., chemicals to be analyzed for) selected for documentation by NOAA's National Status and Trends (S&T) Program.

KEYWORDS: *Organic compounds, *Chemical analysis, *Standards, *Water pollution, *Environmental impacts, *Toxic substances, *Water pollution detection.

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STANDARD ANALYTICAL PROCEDURES

OF THE

NOAA NATIONAL ANALYTICAL FACILITY, 1984-85

EXTRACTABLE TOXIC ORGANIC COMPOUNDS

Prepared for

The NOAA National Status and Trends Program

By

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October 1984

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PREFACE

The analytical procedures for marine environmental samples described herein result from eight years of methods development and application by the National Analytical Facility (NAF) of the National Oceanic and Atmospheric Administration (NOAA). These procedures are published for use in the National Status and Trends (S&T) Program of NOAA's National Ocean Service.

Begun in 1984, the S&T Program seeks to document and assess the present status and future trends of environmental quality throughout the nation's coasts and estuaries. Basically, the S&T Program asks:

What are the current conditions of the nation's coastal zone?

Are these conditions getting better or worse?

To answer such questions the S&T Program will employ a nationally uniform set of environmental measurements. To help ensure that uniformity, this Technical Memorandum documents the analytical procedures for the extractable toxic organic chemicals.

The National Status and Trends Program consists of the following major components:

- The National Benthic Surveillance Project
- The National "Mussel Watch" Project
- The Water Quality Monitoring Project
- The Synthesis of Historical and New Data

This publication is a laboratory manual for use by analytical chemists working on the first two components. The National Benthic Surveillance Project will be conducted by NOAA's National Marine Fisheries Service (NMFS). Under this project NMFS chemists will measure toxic chemicals in bottom sediments and in the fish associated with those sediments. Samples will come from about 150 coastal and estuarine stations, predominantly in urban, industrial areas, but with nonurban areas included for reference. In the National "Mussel Watch" Project, laboratories under contract to NOAA will analyze mussels and other bivalves for the same toxic chemicals planned for the National Benthic Surveillance Project. These molluscs will come from about 150 coastal and estuarine sites nationwide.

For further information on NOAA's National Status and Trends Program write: NOAA/National Ocean Service, N/OMA32, Rockville, MD 20852.

INTRODUCTION

Numerous recent studies demonstrate associations between organic chemical contamination of the aquatic environment and impacts on environmental health and, potentially, on human health (cf. Malins et al. 1984). If the results of one study are to be compared with those of another, uniform analytical methods for the chemicals will be required. To meet this need, NOAA's National Analytical Facility (NAF) prepared this Technical Memorandum as a methods manual for extractable organic chemicals in marine sediments and tissues. It applies specifically to the organic analytes (i.e., chemicals to be analyzed for) selected for documentation by NOAA's National Status and Trends (S&T) Program (Table 1).

The analytical procedures for the organic analytes listed in Table 1 are, for a host of reasons, lengthy and complex. Hence, it is important that the laboratories participating in the S&T Program have specific analytical procedures -- procedures that are described in the detail shown here. This manual is primarily for use by analytical chemists of the National Marine Fisheries Service participating in the National Benthic Surveillance Project. However, it may also be used by laboratories under contract to NOAA for the National "Mussel Watch" Project. Applications to other analogous purposes are welcome, as are suggestions and comments.

NOAA National Analytical Facility

Since, its inception in 1976, NOAA's National Analytical Facility has been at the forefront in developing and employing advanced methods' to analyze aquatic samples for traces of toxic chemicals. These activities have focused primarily on methods for determining industry-related organic compounds such as aromatic hydrocarbons and chlorinated hydrocarbons in both sediments and organisms. Most of the analytes are listed among the EPA-NRDC "Priority Pollutants" (Environmental Protection Agency 1979).

Over the years NAF methods have found wide application in environmental studies concerning the Strait of Juan de Fuca (MacLeod et al. 1977, Brown et al. 1979), the New York Bight (MacLeod et al. 1981), and Puget Sound (Malins et al. 1980, 1982), among others. As the methods are neither simple nor inexpensive, it is most important that the soundest analytical techniques available be employed and that improvements be continually sought. Thus, evolution of the methodology is assured, and this manual will be updated periodically as methods improve.

Quality of Analytical Data

Horwitz and coworkers(1980) observed that the uncertainty in the analytical results in interlaboratory comparisons increases in a regular progression as the concentrations of the particular analyte descend from fractions of a percent to parts-per-million (ppm) to parts-per-billion (ppb) According to their studies, standard deviations (s) for interlaboratory comparisons of means (x) around 10 ppb should not be

Table 1. Extractable organic chemicals and internal standards selected for documentation by NOAA's National Status and Trends Program.

aromatic hydrocarbons (AHs)	chlorinated compounds	
naphthalene	hexachlorobenzene (HCB	
2-methylnaphthalene	lindane (Y-BHC)	
1-methylnaphthalene	heptachlor	
biphenyl	heptachlor epoxide	
2,6-dimethylnaphthalene	aldrin	
acenaphthene	dieldrin	
fluorene	a-chlordane	
phenanthrene	trans-nonachlor	
anthracene	mirex	
1-methylphenanthrene		
fluoranthene	o,p-DDE	DDTs
pyrene	p,p-DDE	
benz[a]anthracene	o,p-DDD	
chrysene	p,p-DDD	
benzo[e]pyrene	o,p-DDT	
benzo[a]pyrene	p,p-DDT	
perylene		
dibenz[a,h]anthracene	dichlorobiphenyls	PCB5
	trichlorobiphenyls	
	tetrachlorobiphenyls	
internal standards (I-Stds)	pentachlorobiphenyls	
	hexachlorobiphenyls	
naphthalene-d_8	heptachlorobiphenyls	
acenaphthene-d_{10}	octachlorobiphenyls	
perylene-d_{12}	nonachlorobiphenyls	
hexamethylbenzene (HMB)		
tetrachloro-m-xylene (TCMX)	natural product (sewage tracer)	
5a-androstan-3-B-ol		
	coprostanol	

expected to be better than 35% of the grand mean (x). Our experience (MacLeod et al. 1982) has shown this thesis to be realistic but it often dismays or confounds statisticians, modelers, and administrators. Nevertheless, the issue must be faced, and the best possible precision must be secured for the analytical results. Accordingly, the methods published here are the best compilation of techniques we could devise.

In implementing these procedures the following quality assurance (QA) protocols will be observed. First the procedures will be validated statistically in NAF's laboratories, consistent with the Horwitz model above. Then NAF will distribute calibrating solutions and previously analyzed sample extracts to the participating NMFS laboratories so that they may test the performance of their measuring equipment, i.e., the gas chromatograph(s). Once consistent and satisfactory results have been obtained, interim reference materials (IRMs) will be supplied to the laboratories for assessing their proficiencies with the analytical procedures. The goal is to have the interlaboratory standard deviations conform as closely as possible to the Horwitz model.

Summary of Analytical Procedures

In general, analyses of sediment and organisms follow the scheme shown in Figure 1, as summarized below:

> Thaw the sample (if frozen), remove excess water, and weigh the sample (ca. 10 g for sediment or 3 g of tissue or fluid) to the nearest 0.01 g. Add the extraction solvent (CH_2Cl_2) and internal standards (I-Stds), then mix/grind/extract sample with sodium sulfate 3x under CH_2Cl_2. Combine the solvent extracts and concentrate them by evaporation with heating. Chromatograph the extract concentrate on Type 923 silica gel and basic alumina, and collect fractions eluted with pentane (fraction SA1) and 50% CH_2Cl_2 in pentane (fraction SA2). In sediments only, continue to elute with CH_2Cl_2 and CH_3OH (fraction SA3). Concentrate fraction SA2 by evaporation with heating, and then chromatograph it on precalibrated Sephadex LH-20. Collect the 2nd fraction from Sephadex chromatography (fraction SA2-L2), and concentrate it to 1 mL if from sediment, or to 0.1 mL if from tissue. Analyze fraction SA2-L2 from sediment and tissue (except liver) for the aromatic hydrocarbons (AHs) in Table 1 (page 2) by capillary gas chromatography (GC) with a flame-ionization detector (FID). Also analyze fraction SA2-L2 from all samples for the chlorinated hydrocarbons on page 2, using an electron-capture detector (ECD). If hexachlorobenzene (HCB) is found, also analyze fraction SA1 for HCB as with fraction SA2-L2. Concentrate fraction SA3 from sediments, and analyze it by GC-FID for coprostanol.

For the convenience of the bench chemists, the procedures summarized above are presented in detailed sections, each of which deals with a major analytical operation. The order of the sections appears on the Contents page.

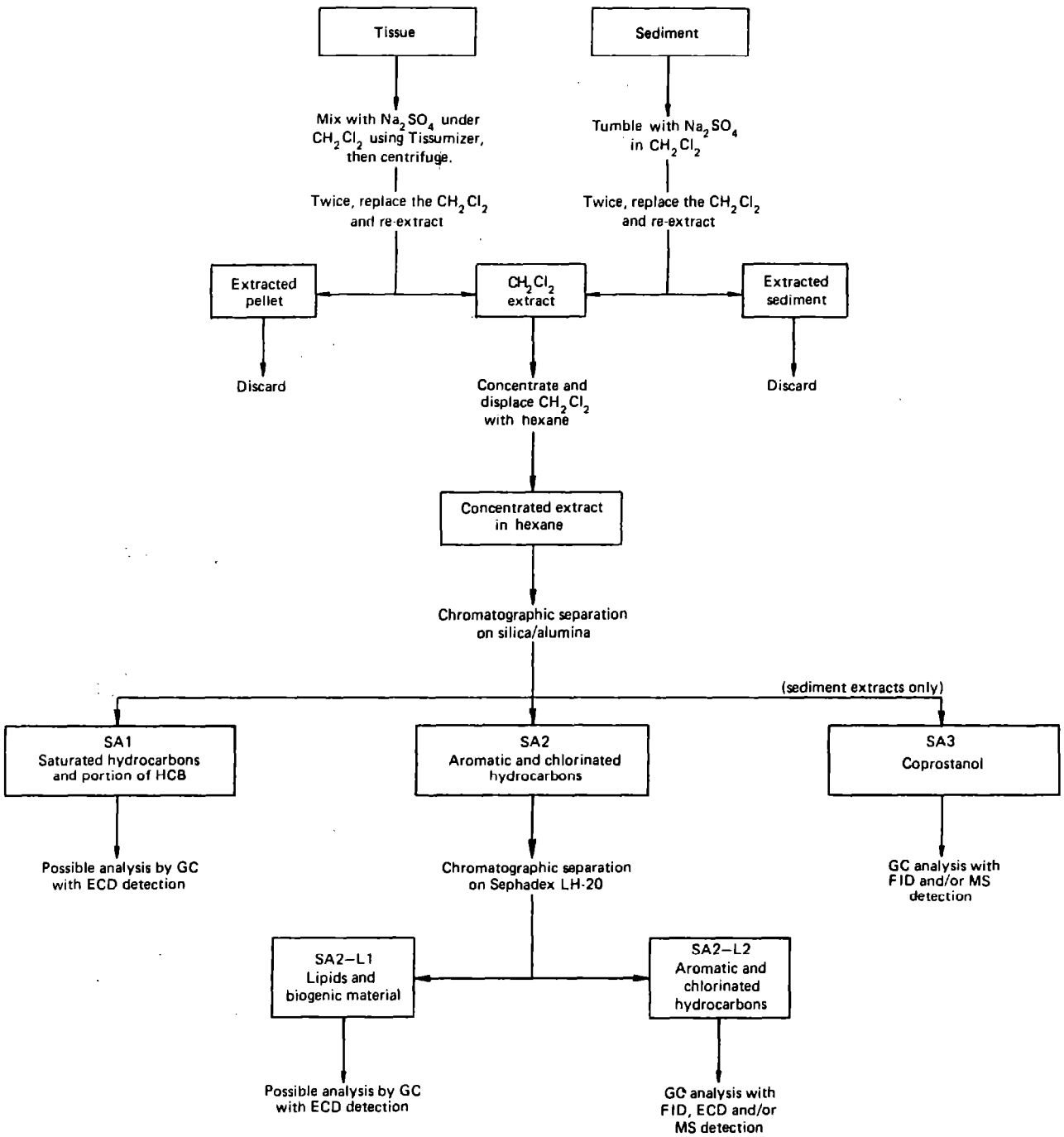

Figure 1. Summary of the analytical procedures of the National Analytical Facility for trace extractable toxic organic analytes.

SECTION 1

MATERIALS

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MATERIALS

Disclaimer: Mention of a product or company name does not imply endorsement by the Department of Commerce to the exclusion of others that may be suitable.

A. Solvents

cyclohexane	Brand: _____, Lot # _____
hexane	Brand: _____, Lot # _____ checked per Section 4E, page 35
wash methanol (CH_3OH)	Brand: _____, Lot # _____ checked per Section 4A, page 25
redistilled CH_3OH	Prepared from wash CH_3OH per Section 2B Note 2, page 17, and checked per Section 4A, page 25
dichloromethane (CH_2Cl_2)	Brand: _____, Lot # _____ checked per Section 4C, page 31
pentane	Brand: _____, Lot # _____ checked per Section 4D, page 33

B. Column Packings

silica gel	Davison Type 923, 100-200 mesh (nominal), accepted lots only, per Section 5
alumina	Baker, basic, Brockman Activity I
size-exclusion gel	Sephadex LH-20 accepted lots only, per Section 6
sand, acid-washed	Ottawa, MCB, kiln-dried, 30-40 mesh (steeped in conc. HCl overnight, then washed with H_2O 3x and stored at 120°C)

C. Reagents

Na_2SO_4	Reagent Grade, anhydrous granular
copper	Reagent Grade, fine granular

D. Miscellaneous

boiling chips	Teflon, Norton Chemplast, Chemware

E. Yellow Laboratory Lighting

Yellow fluorescent and/or incandescent lights

Yellow transparent acetate sheeting on windows

F. Standards

HMB GC I-Std solution, ca. 100 ng/μL of hexamethylbenzene in hexane

Actual conc.: Std #_____, ng/μL _____; Std # _____, ng/μL _____

TCMX GC I-Std solution, ca. 2 ng/μL of tetrachloro-m-xylene in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

AH I-Std solution, ca. 50 ng/μL of each I-Std in hexane

Actual conc.:	Std # _____	Std # _____
naphthalene-d_8	ng/μL _____	ng/μL _____
acenaphthene-d_{10}	" _____	" _____
perylene-d_{12}	_____	_____

PES I-Std solution, ca. 1 ng/μL of _____ in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

COP I-Std solution, ca. 50 ng/μL of 5α-androstan-3β-ol in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

COP GC-calibration check solution, ca. 5 ng/uL in hexane of:

Actual conc.:	Std # _____	Std # _____
5α-androstan-3β-ol	ng/μL _____	ng/μL _____
coprostanol	" _____	" _____

COP spike solution, ca. 50 ng/μL of coprostanol in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

F. Standards (continued)

AH GC-calibration check solution, ca. 5 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
hexamethylbenzene (GC I-Std)	ng/µL _____	ng/µL _____
naphthalene	" _____	" _____
2-methylnaphthalene	_____	_____
1-methylnaphthalene	_____	_____
biphenyl	_____	_____
2,6-dimethylnaphthalene	_____	_____
acenaphthene	_____	_____
fluorene	_____	_____
phenanthrene	_____	_____
anthracene	_____	_____
1-methylphenanthrene	_____	_____
fluoranthene	_____	_____
pyrene	_____	_____
benz[a]anthracene	_____	_____
chrysene	_____	_____
benzo[e]pyrene	_____	_____
benzo[a]pyrene	_____	_____
perylene	_____	_____
dibenz[a,h]anthracene	_____	_____
naphthalene-d_8 (I-Std)	_____	_____
acenaphthene-d_{10} (I-Std)	_____	_____
perylene-d_{12} (I-Std)	_____	_____

F. Standards (continued)

PES GC-calibration check solution, ca. 0.1 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
tetrachloro-m-xylene (GC I-Std)	ng/µL _____	ng/µL _____
hexachlorobenzene	" _____	" _____
lindane (γ-BHC)	_____	_____
heptachlor	_____	_____
heptachlor epoxide	_____	_____
aldrin	_____	_____
α-chlordane	_____	_____
trans-nonachlor	_____	_____
dieldrin	_____	_____
mirex	_____	_____
o,p'-DDE	_____	_____
p,p'-DDE	_____	_____
o,p'-DDD	_____	_____
p,p'-DDD	_____	_____
o,p'-DDT	_____	_____
p,p'-DDT	_____	_____
dichlorobiphenyls	_____	_____
trichlorobiphenyls	_____	_____
tetrachlorobiphenyls	_____	_____
pentachlorobiphenyls	_____	_____
hexachlorobiphenyls	_____	_____
heptachlorobiphenyls	_____	_____
octachlorobiphenyls	_____	_____
nonachlorobiphenyls	_____	_____
_____ (I-Std)	_____	_____

F. Standards (continued)

AH spike solution, ca. 50 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
naphthalene	ng/µL _____	ng/µL _____
2-methylnaphthalene	" _____	" _____
1-methylnaphthalene	_____	_____
biphenyl	_____	_____
2,6-dimethylnaphthalene	_____	_____
acenaphthene	_____	_____
fluorene	_____	_____
phenanthrene	_____	_____
anthracene	_____	_____
1-methylphenanthrene	_____	_____
fluoranthene	_____	_____
pyrene	_____	_____
benz[a]anthracene	_____	_____
chrysene	_____	_____
benzo[e]pyrene	_____	_____
benzo[a]pyrene	_____	_____
perylene	_____	_____
dibenz[a,h]anthracene	_____	_____

F. Standards (continued)

PES spike solution, ca. 1 ng/µL in hexane of:

Actual conc.:	Std # ____	Std # ____
hexachlorobenzene (HCB)	ng/µL ____	ng/µL ____
lindane (γ-BHC)	" ____	" ____
heptachlor	____	____
heptachlor epoxide	____	____
aldrin	____	____
α-chlordane	____	____
trans-nonachlor	____	____
dieldrin	____	____
mirex	____	____
o,p'-DDE	____	____
p,p'-DDE	____	____
o,p'-DDD	____	____
p,p'-DDD	____	____
o,p'-DDT	____	____
p,p'-DDT	____	____
dichlorobiphenyls	____	____
trichlorobiphenyls	____	____
tetrachlorobiphenyls	____	____
pentachlorobiphenyls	____	____
hexachlorobiphenyls	____	____
heptachlorobiphenyls	____	____
octachlorobiphenyls	____	____
nonachlorobiphenyls	____	____

SECTION 2

6:4 CYCLOHEXANE:METHANOL AZEOTROPE PREPARATION

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6:4 CYCLOHEXANE:METHANOL AZEOTROPE PREPARATION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the solvents.

Glassware

22-L round bottom boiling flask with a 24/40-STJ port, a 71/60-STJ port, and a thermometer well filled with corn oil

22-L round bottom receiver flask with a 45/50-STJ port

5-L round bottom receiver flask with a 24/40-STJ port

2-L TC graduated cylinder

ZOO-mm OD, long-stem funnel

adapter, 45/50-STJ to 24/40-STJ

other distillation apparatus (24/40-STJ): fractionation column (5 cm x 50 cm, packed with 7-mm lengths of 6-mm glass tubing), stillhead with 10/30-STJ thermometer port, condenser (Corning 2400 or Kimble 18140), 3-way receiver valve (8,mm bore Teflon stopcock), misc. fittings

Solvents

10 L cyclohexane

8 L washing methanol

Other Materials and Apparatus

heating mantle for 22-L flask

Variac transformer

2 ea automatic temperature controllers (YSI Models 63RC and 74)

timing clock

Hoffman clamp

boiling chips

2 ea Lab Jax

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Wash all glassware, including the distillation apparatus, twice with CH_2Cl_2 before each run.
2. Attach the 22-L receiver flask to the front receiver-valve port. Attach the 5-L flask to the rear receiver-valve port, and set the receiver valve to collect the distillate in it.
3. Put the 22-L boiling flask in the heating mantle. Place the stillhead into the top of the fractionation column, and fit the column into the 24/40-STJ port of the boiling flask. Align the stillhead outlet and the condenser inlet-fittings, then secure the ball and socket joint with the Hoffman clamp.
4. Wash-the glass sensor probe of the Model 74 temperature controller twice with CH_2Cl_2, and set it firmly into the 10/30-STJ port at the top of the stillhead. Also, check that the thermometer well of the boiling flask is filled to a depth of 2 cm with corn oil, then insert the metal probe of the Model 63RC temperature controller.
5. Place a large funnel in the 71/60-STJ port, then fill the boiling flask with 10 L of cyclohexane and 8 L of CH_30H i.e., a small excess of CH_30H). Add 40-50 boiling chips, and stopper the flask.
6. Turn on the condenser cooling water. Set the Variac at 60, the Model 74 temperature controller at 55.5°C, and the Model 63RC temperature controller at 68°C. Start the distillation by switching on the timer.
7. Collect 3 L of 6:4 azeotrope forerun (during 6 hr) in the 5-L receiver flask (see Note B.1, page 17). Then switch the receiver

B. Procedure (continued)

valve to collect most of the distillate in the 22-L receiver. during 24 hr. As the solvent temperature in the boiling flask rises toward 65°C, distillation slows. Only a small amount. (ca. 1 L), mostly CH_3OH, remains undistilled, so switch the timer off to stop the distillation.

8. Allow the distillation apparatus to cool, then disassemble it. Discard the boiling chips, and set the undistilled solvent aside for recycling (Note 1). Wash the apparatus and flasks twice with CH_2Cl_2. Reassemble as before, pouring only the 6:4 azeotropic distillate (a 2-phase mixture) from the large receiver back into the boiling flask. Add 40-50 boiling chips.
9. Make sure that the cooling water is flowing, then switch timer on, and distill 1 L of forerun into the 5-L flask (see Note B.1). Switch the receiver valve so as to collect most of the distillate in the 22-L receiver. Distill until the solvent level (ca. 1 L) reaches the bottom of the thermometer well. Switch off the timer.
10. Allow the apparatus to cool. Remove the large receiver. Discard the boiling chips and set the undistilled solvent aside for recycling (Note 1).
11. Proceed to Section 3 (page 19) with the 6:4 azeotrope.

Notes

1. These boiling flask residues and foreruns may be saved and recycled into step 5 of a subsequent distillation. However, they should not be recycled more than twice.
2. Redistilled CH_3OH is prepared from the 6:4 azeotrope by (a) adding 1/lOth volume of carbon-filtered, distilled H_2O to the

B. Procedure (continued)

azeotrope from step 7, (b) allowing the phases to separate in a separatory funnel, (c) draining the lower phase into a distilling flask, and (d) distilling pure CH_3OH through a fractionation column. Check the purity per Section 4A, page 25. The upper phase remaining in the separatory funnel, mostly cyclohexane, can be recycled through the 6:4 Azeotrope Preparation in step B.5, page 16.

SECTION 3

PREPARATION OF 6:4:3 SOLVENT

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PREPARATION OF 6:4:3 SOLVENT

A. Equipment List- Note:. CH_2Cl_2-wash all of the glassware and materials contacting the distilled solvent.

Glassware

2-L TC graduated cylinder

ZOO-mm OD, long-stem funnel

20-L carboy

50-mL volumetric pipet

1-L 24/40-STJ Erlenmeyer flask with stopper

4-L standard solvent bottles

Solvents

6:4 cyclohexane:methanol azeotrope (from Section 2, step B.lO, page 17)

CH_2Cl_2

Other Materials and Apparatus

pipet filler, 3-valve, rubber (for volumetric pipet)

Teflon-lined stopper for carboy

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Prepare a sample of 6:4:3 solvent for purity testing by pipetting 200 mL each of the upper and lower layers of the 6:4 azeotrope (from Section 2, step B.10, page 17) into the flask.
2. Add 120 mL of CH_2Cl_2 to the flask and mix well. Check the purity of the solvent by proceeding with this sample to Section 4B, page 29.

B. Procedure (continued)

3. If the purity of the sample from step 2 is acceptable according to Section 4B (page 29), proceed to step 4. Otherwise, return the remaining 6:4 azeotrope to the boiling flask in Section 2, step B.5 (page 16) for redistillation.
4. Transfer the remaining 6:4 azeotrope into the carboy in 2000-mL increments.
5. Note the total volume of the 6:4 azeotrope, and multiply it by 0.30. This is the volume of CH_2Cl_2 to be added to the 6:4 azeotrope to make the 6:4:3 solvent.
6. Add the amount of CH_2Cl_2 calculated in step 5 to the carboy.
7. Stopper the carboy, and mix the 6:4:3 solvent until it is completely homogeneous.
8. Transfer the 6:4:3 solvent into 4-L solvent bottles for storage until use in Section 6 (page 45) or in Section 11 (page 77).

SECTION 4

TESTING SOLVENTS FOR PURITY*

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* Criteria: When a solvent sample is analyzed by GC (Section 12, Page 85), no GC peaks should occur within 0.1 min of an analyte peak and no peaks occurring after the retention time of o-xylene or hexachlorobenzene should give a chart deflection > 5%.

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A. REDISTILLED CH_3OH PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the CH_3OH sample.

Glassware

lOO-mL TC graduated cylinder

3 ea 500-mL separatory funnels

6 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials

Solvents

200 mL redisti lled CH_3OH from Section 2, Note B.2, page 17

135 mL CH_2Cl_2 (not including washes)

1500 mL carbon-filtered, distilled H_2O

GC Standard Solution (per sample)

50,µL HMB GC I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (block contains: Al inserts fit to the 0.7 mL line of the tube tip, and an Al-foil shroud, with TLC plate window, 5 cm taller than tubes in block)

100-µL syringe

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for CH_3OH - Note: Perform in duplicate.

a. Extraction

(1) Add 100 mL of redistilled CH_3OH (Section 2, Note B.2, page 17) and 25 mL of CH_2Cl_2 to a separatory funnel. Swirl the funnel for a few seconds to mix well.

(2) Add 250 mL of carbon-filtered, distilled H_2O to the separatory funnel, and shake it vigorously for 2 min. Allow the phases to separate well.

(3) Drain the lower phase into a flask, leaving behind any emulsion layer. Save the contents of the flask.

(4) Add 10 mL of CH_2Cl_2 to the separatory funnel, and shake it vigorously for 2 min. Allow the phases to separate well.

(5) Drain the lower phase into the flask from step 3, including any emulsion layer.

(6) Discard the contents of the separatory funnel.

(7) Pour the extract from the flask back into the separatory funnel.

(8) Wash the flask with 3-4 mL of CH_2Cl_2, and add the washings to the separatory funnel.

(9) Repeat step (8) once. This flask is no longer needed.

(10) Repeat steps (2)-(6), EXCEPT use a fresh flask in step (3), and do not include the emulsion layer in step (5).

b. Concentration

(1) Add 3-4 boiling chips to the flask from step 2.a(10), and attach a Snyder column.

(2) Concentrate the extract in a 60°C water bath to 10-15 mL.

(3) Transfer the extract to a labeled concentrator tube (no CH_2Cl_2 washes!).

b. Concentration (continued)

(4) Add a boiling chip to the tube and, using the tube heater, concentrate the sample to ³ 0.9 mL, < 1.0 mL.

(5) Add 50 µL of HMB GC I-Std solution to the extract, and mix on the Vortex Genie for 2 sec at setting 8-10.

(6) Transfer the extract to a labeled GC vial, and cap the vial.

(7) Proceed to GC Analysis (Section 12, page 85).

3. Procedure for CH_3OH Blank

a. Proceed as in Subsection 2 above, except omit the 100 mL of CH_3OH in step 2.a(1), and perform only a single analysis.

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B. 6:4:3 SOLVENT PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the solvent sample.

Glassware

100-mL graduated cylinder

2 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

2 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials

Solvents

200 mL 6:4:3 solvent from Section 3, step B.2, page 21

3 mL redistilled CH_3OH

21 mL hexane

GC Standard Solution (per sample)

50 µL HMB GC I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, page 25)

100-µL syringe

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for 6:4:3 solvent - Note: Perform this analysis in duplicate.

   a. Transfer 100 mL of the 6:4:3 solvent from Section 3, step B.2, page 21, to a flask.

   b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

   C. Concentrate the sample in a 75°C water bath to 10-15 mL.

   d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

   e. Add a boiling chip and 1 mL of redistilled CH_3OH to the tube, and using the tube heater, concentrate the sample to ³ 0.9 mL, < 1.0 mL.

   f. Add 7 mL of hexane to the tube, and concentrate the sample to ³ 0.9 mL, < 1.0 mL.

   g. Add 50 µL of HMB GC I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting 8-10.

   h. Transfer the sample to a GC vial, cap the vial, and label it.

   i. Proceed to GC Analysis (Section 12, page 85).

3. Procedure for a Blank

   a. Prepare a blank by adding 1 mL of redistilled CH_3OH and 7 mL of hexane to a tube.

   b. Concentrate the solvents to ³ 0.9 mL, < 1.0 mL.

   c. Proceed as in steps 2.g-2.i above.

C. CH_2Cl_2 PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the CH_2Cl_2 sample.

Glassware

2 ea 500-mL TC graduated cylinders

3 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials

Solvents

700 mL CH_2Cl_2 from lot to be tested

350 mL CH_2Cl_2 from lot currently in use

GC Standard Solution (per sample)

50 µL HMB GC I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, page 25)

100-µL syringe

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for CH_2Cl_2 - Note: Perform a duplicate analysis for the lot to be tested, and perform a single analysis with a CH_2Cl_2 lot currently in use-.

   a. Add 350 mL of CH_2Cl_2 to a flask.

   b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

   C. Concentrate the sample in a 60°C water bath to 10-15 mL.

   d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

   e. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

   f. Add 50 µL of HMB GC I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting 8-10.

   g. Transfer the sample to a labeled GC vial, and cap the vial.

   h. Proceed to GC Analysis (Section 12, page 85).

D. PENTANE PURITY

1. Equipment List - Note: CH_2l_2-wash all glassware and materials contacting the pentane sample.

Glassware

2 ea 100-mL TC graduated cylinders

3 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials

Solvents

200 mL pentane from lot to be tested

100 mL pentane from lot currently in use

GC Standard Solution (per sample)

50 µL HMB GC I-Std solution

Other Materials and Apparatus

boiling chips

water bath

modified Kontes tube heater (Section 4A, page 25)

100-µL syringe

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for Pentane - Note: Perform duplicate analyses for the lot to be tested, and a single analysis with a pentane lot currently in use.

a. Add 100 mL of pentane to a flask.

b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

C. Concentrate the sample in a 55°C water bath to 10-15 mL.

d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

e. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to ³ 0.9 mL, < 1.0 mL.

f. Add 50 µL of HMB GC I-Std solution to the sample, and mix on a Vortex Genie for 2 sec at setting 8-10.

g. Transfer the sample to a labeled GC vial, and cap the vial.

h. Proceed to GC Analysis (Section 12, page 85).

E. HEXANE PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the hexane sample.

Glassware

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials

Solvents

50 mL hexane from lot to be tested

25 mL hexane from lot currently in use

GC Standard Solution (per sample)

50 µL HMB GC I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, page 25)

100-µL syringe

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for Hexane - Note: Perform duplicate analyses for lot to be tested, and a single analysis with a hexane lot currently in use.

   a. Add 25 mL of hexane to a concentrator tube.

   b. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

   C. Add 50 µL of HMB GC I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting B-10.

   d. Transfer the sample to a labeled GC vial, and cap the vial.

   e. Proceed to GC Analysis (Section 12, page 85).

SECTION 5

LOT TESTING/CALIBRATION OF SILICA GEL/ALUMINA

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LOT TESTING/CALIBRATION OF SILICA GEL/ALUMINA

A. Equipment List - Note: CH_2Cl_2-wash all of glassware and materials contacting the silica gel.

Glassware (per column calibrated).'

19-mm ID x 30-cm chromatography column with reservoir

2 ea 250-mL beakers

50-mL TC graduated cylinder

3 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

22 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

22 ea 2-mL GC vials

transfer pipets (Pasteur style) with rubber bulbs.

Reagents and Solvents (per column calibrated)

20 g silica gel (heated to 700°C for 18 hr, stored at 17O°C, and cooled to room temp. in a desiccator just before use)

10 g alumina (activated at 170°C for 18 hr, then cooled to room temp. in a desiccator just before use)

7.5 cc activated copper (< 1 hr before use, activate copper by covering it with conc. HCl and stirring with a glass rod, then allowing it to stand for 5 min, followed by washing twice with CH_3OH and then 3x with CH_2Cl_2)

ca. 1 cc sand

100 mL pentane

200 mL 1:l CH_2Cl_2:pentane (v:v)

210 mL CH_2Cl_2 (not including washes)

25 mL 10% redistilled CH_3OH in CH_2Cl_2 (v:v)

25 mL 20% redistilled CH_3OH in CH_2Cl_2 (v:v)

A. Equipment List (continued)

Standards and Calibrating Solutions

50 µL HMB GC I-Std solution (per SA fraction)

50 µL TCMX GC I-Std solution (per. SA fraction)

silica-gel calibrating solution: Extract 10 samples each of control (relatively clean) sediment and of control mussel, per Sections 7 and 8, respectively. Combine the 20 extracts (2 mL each), and add: (a) 10 mL of AH spike solution, (b) 1 mL of PES spike solution, and (c) coprostanol to give a final concentration of ca. 2 µg/mL (final volume ca. 55 mL).

Other Materials and Apparatus

desiccator

curved-stem funnel (curve glassblown)

powder funnel

2500-µL and 100-µL syringes

glass wool and glass rod

boiling chips

modified Kontes tube heater (Section 4A, page 25)

water bath

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Column Preparation

1. Prepare the columns just prior to use. On hot days elute more slowly to maintain the column integrity.
2. Fit a 19-mm ID column with a stopcock, add 100 mL of CH_2Cl_2 and a 5-15-mm glass-wool plug. Tamp the plug well to remove any bubbles.
3. Add the alumina to a beaker, and slowly add 20 mL of CH_2C1_2. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 6.

B. Column Preparation (continued)

4. Add the silica gel to a 2nd beaker. Slowly add 40 mL of CH_2Cl_2 to the beaker. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 7.
5. Place a curved-stem funnel into the column reservoir so that the funnel tip hangs well off-center.
6. Swirl the beaker to resuspend the alumina from step 3, and pour the slurry into the column.
7. Wash the beaker with ca. 5 mL of CH_2Cl_2, and add the washings to the column. Repeat the wash twice.
8. After the particles settle, open the stopcock for 30 sec to allow the alumina to pack more tightly, then close the stopcock.
9. Add the silica gel from step 4 to the column, as in steps 6-7 for the alumina.
10. Place the beaker under the column tip, and after the silica gel has-settled, open the stopcock. While the solvent is still draining, add the sand and then the copper through the powder funnel. Drain to the packing top, then close the stopcock.
11. Add 50 mL of pentane to the column. Drain to the packing top, then close the stopcock. Discard the eluates collected thus far.

C. Calibrating the Column

1. Using the 2500 µL syringe, place 2 mL of the silica gel calibration solution on top of the packing.
2. Place a cylinder, labeled “SA1.1”, beneath the column.
3. Open the stopcock; and drain to the packing top, then close the stopcock.

C. Calibrating the Column (continued)

4. From the remaining 50 mL of pentane, add 0.5 mL of pentane to the packing. Open the stopcock. Drain to the packing top, then close the stopcock.
5. Repeat step 4 once.
6. Add the rest of the pentane to the column, and elute at ca. 2 mL/min until 35 mL has been collected in the cylinder. Close the stopcock.
7. Pour the eluate in the cylinder labeled "SA1.1" into a similarly labeled flask.
8. Wash the cylinder with 3-4 mL of CH_2Cl_2, and add the washings to the flask.
9. Repeat step 8 once.
10. Replace the cylinder with a concentrator tube labeled "SA1.2", and collect 2.0 mL. Close the stopcock.
11. Using tubes labeled successively "SA1.3"-"SAl.11", repeat step 10 9 times, adding 200 mL of 1:1 (v:v) CH_2C1_2:pentane to the column when the pentane in the column drains to the packing top.
12. Replace the last tube from step 11 with a tube labeled "SA2.1", and collect 20 mL of eluate. Close the stopcock.
13. Using tubes labeled successively "SA2.2"-"SA2.10", repeat the process in step 12 9 times, adding 50 mL of CH_2Cl_2 when the CH_2Cl_2:pentane in the column drains to the packing top.
14. Replace the last tube in step 13 with a "waste" flask, and drain the remaining solvent to the packing top.
15. Add 25 mL of 10% CH_3OH in CH_2Cl_2 to the column, and drain to the packing top. Discard the contents of the waste flask.

C. Calibrating the Column (continued)

16. Label a flask "SA3", and replace the waste flask with it.
17. Add 25 mL of 20% CH_3OH in CH_2Cl_2 to the column, and elute all of it into the SA3-labeled flask.

D. Concentration of Fractions

1. Add 3-4 boiling chips, and attach a Snyder column to each flask from steps C.9 and C.17.
2. Concentrate each fraction in a 55°-60°C water bath to 10-15 mL.
3. Transfer each fraction from step 2 to a labeled tube.
4. Wash each flask with 3-4 mL of CH_2Cl_2, and add the washings to the corresponding tube.
5. Repeat step 4 once.
6. Add a boiling chip to each tube from steps C.10-D.5, and using the tube heater, concentrate each fraction to > 0.9 mL, < 1.0 mL.
7. Add 2 mL of hexane to each tube, and concentrate each fraction to > 0.9 mL, < 1.0 mL.
8. Add 50 µL of HMB GC I-Std solution and 50 µL of TCMX GC I-Std solution to each fraction, and mix each on the Vortex Genie for 2 sec at setting 8-10.
9. Transfer each fraction to a labeled GC vial, cap the vial, and analyze by GC-FID and GC-ECD (Section 12, page 85).
10. From the analyses, determine where the fraction cuts should be made.

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SECTION 6

SEPHADEX LH-20 COLUMN PREPARATION AND CALIBRATION

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SEPHADEX LH-20 COLUMN PREPARATION AND CALIBRATION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample.

Glassware (per column calibrated with azulene/perylene)

19-mm ID x 30-cm chromatography column with reservoir

100-mL TC graduated cylinder

Additional Glassware (per column calibrated with "calibration extract")

2 ea 50-mL TC graduated cylinders

2 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

22 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

22 ea 2-mL GC vials

transfer pipets (Pasteur style) with rubber bulbs

Reagents and Solvents (per column calibrated with azulene/perylene)

80 cc wet Sephadex LH-20 (swelled overnight in 6:4:3 solvent), plus 50 mL additional 6:4:3 solvent

2.5 cc sand

350 mL 6:4:3 solvent

Additional Reagents and Solvents (per column calibrated with "calibration extract")

200 mL 6:4:3 solvent

hexane and redistilled CH_3OH (as needed)

Standards and Calibrating Solutions

50 µL HMB GC I-Std solution (per fraction)

50 µL TCMX GC I-Std solution (per fraction)

Azulene/perylene calibrating solution: Add enough azulene (ca. 10 mg/mL) and perylene (ca. 1 mg/mL) to the 6:4:3 solvent to produce a deeply colored solution. Make sure that the azulene and perylene are completely dissolved.

A. Equipment List (continued)

Standards and Calibrating Solutions (Continued)

Sediment/tissue-extract calibrating solution ("calibration extract"): Extract 10 samples each of control sediment and of control mussel tissue, per Sections 7 and 8, respectively. Chromatograph these samples on silica gel/alumina, per Section 10. Combine the 20 SA2 fractions (2 mL each) from Section 10, step E.2, page 75, and add: (a) 1 mL of PES spike solution, and (b) 10 mL of mL of AH spike solution to the combined fractions. Concentrate this to 10 mL, and add sufficient CH_3OH and CH_2Cl_2 to make a 6:4:3 hexane:CH_3OH:CH_2Cl_2 solution.

Other Materials and Apparatus

curved-stem funnel (curve glassblown)

glass wool

UV light (UVS•11 Mineralight)

2500-µL and 100-µL syringes

boiling chips

modified Kontes tube heater (Section 4A, page 25)

waterbath

Vortex Genie

75-cm x 6-mm glass rod

aluminum foil

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Column Preparation

1. Fit a 19-mm ID column with a stopcock, add 10 mL of 6:4:3 solvent. and a 5-10-mm glass-wool plug. Tamp the plug to remove any air bubbles.
2. Add ca. 1 cc of sand to the column, and tap the column gently so that it forms a smooth layer on top of the glass wool.
3. Pour the swelled Sephadex gel through a curved-stem funnel into the column until the gel fills the column and about l/4 of the reservoir.

B. Column Preparation (continued)

4. Allow 10 min for the Sephadex to settle. Open the stopcock, and collect 80 mL to ensure firm packing. Add more solvent as needed. Leave 30 mL of solvent in the column reservoir. Cover the top with aluminum foil, and allow the packing to settle overnight.
5. Open the stopcock, and elute 10 mL of solvent, then close the stopcock. Remove the excess Sephadex packing from the top with a transfer pipet until the height of the Sephadex is 26.5 cm.
6. Gently add ca. 1 cc of sand onto the packing so that it forms an even layer on the top. (The column may be tapped or tilted slightly to get an even layer of sand.)
7. Examine the packing for air bubbles. If bubbles are evident, elute ca. 250 mL of warm (ca. 35°C) solvent through the column. If the bubbles persist, recycle the packing.

C. Column Calibration with Azulene/Perylene (All Columns)

1. Place a 100-mL cylinder beneath the column.
2. Using a transfer pipet, carefully remove any excess 6:4:3 solvent from the top of the packing.
3. Using care not to disturb the packing, place 2 ml of the azulene/perylene calibrating solution on top of the packing.
4. Open the stopcock, drain to the packing top, and close the stopcock.
5. Add ca. 0.5 mL of solvent to the top of the column. Drain to the packing top, and close the stopcock.
6. Repeat step 5 once.
7. Add 100 mL of solvent, and open the stopcock.

C. Column Calibration with Azulene/Perylene (All Columns) (continued)

8. Elute the solvent until all of the perylene has emerged, using the UV light to monitor the perylene. Record the volumes at which the azulene and perylene start and finish eluting.

9. If the azulene emerges in the 50-65 mL range, and the perylene emerges in the 60-80 mL range without distinct tailing on the packing, proceed to step 10. Otherwise, discard the packing.

10. Discard the eluate. Add 50 mL ofsovent to the column, and flush the packing by eluting 50 mL into the cyinder. Again, discard the eluate.

11. The column is now ready for the next sample.

NOTE: If the column is to be stored, maintain 30-50 mL of solvent in the column reservoir, and cover the top with aluminum foil. Remove the solvent if it separates into 2 phases, add 80 mL fresh solvent, and elute 50 mL.

D. Column Calibration with Calibration Extract

1. Set aside one representative column for every 10 columns made, remove any excess 6:4:3 solvent with a transfer pipet.

2. Wash the column tip with CH_2Cl_2, and place a 50-mL cylinder labeled "L1.0" under the column.

3. Place 2 mL of calibration extract on top of the packing. Drain to the packing top, and close the stopcock.

4. Add ca. 0.5 mL of solvent to the column. Drain to the packing top, and close the stopcock.

5. Repeat step 4 once.

6. Add 200 mL of solvent to the column, and collect 30 mL of eluate in the cylinder. Close the stopcock, and transfer the eluate to a flask labeled "L1.0".

7. Wash down the cylinder with 3-4 mL of CH_2C1_2, and add the washings to the flask. Repeat once, and set the flask aside for step E.1.

D. Column Calibration with Calibration Extract (continued)

8. Place a concentrator tube labeled "L1.1" under the column, and collect 1.0 mL of eluate.

9. Repeat step 8 14 times, labeling) the successive fractions "L1.2" through “L1.15”) respectively.

10. Replace the last tube with a 50-mL cylinder labeled "L2.0", and collect 50 mL of eluate. Close the stopcock, and transfer the eluate to a flask labeled "L2.0".

11. Wash down the cylinder with 3-4 mL of CH_2Cl_2, and add the washings to the flask. Repeat once, and set the flask aside for step E.1.

12. Place a concentrator tube labeled "L2.1" under the column, and collect 10 mL of eluate.

13. Repeat step 12 4 times, labeling the successive fractions "L2.2" through "L2.5", respectively.

14. Replace tube "L2.5" with the 100-mL "waste" cylinder (from step C.10), and flush the packing by eluting 50 mL of solvent. Discard this eluate.

E. Concentration of Fractions

1. Add 3-4 boiling chips to the flasks from steps D.7 and D.ll, attach Snyder columns, and concentrate the fractions in a 75°C water bath to 10-15 mL.

2. Transfer the fractions L1.0 and L2.0 to correspondingly-labeled concentrator tubes with 2 washes of 3-4 mL of CH_2Cl_2 each.

3. Add 1 mL of CH_3OH and a boiling chip to each tube (steps D.8-E.2), and concentrate each fraction to > 0.9, < 1.0 mL on the tube heater.

4. Add 7 mL of hexane to each tube, and concentrate to > 0.9, < 1.0 mL.

E. Concentration of Fractions (continued)

5. Add 50 µL of HMB GC I-Std solution and 50 µL of TCMX GC I-Std solution to each fraction.
6. Mix each fraction on the Vortex Genie for 2 sec at setting 8-10.
7. Transfer each fraction to a GC vial for analysis by GC-FID and GC-ECD (Section 12, page 85).
8. After verification of column peformance by GC analysis (i.e., separation of analytes from lipid material), the correct elution volumes can be assigned by choosing those volumes which leave all analytes of interest in the L2 fraction.

SECTION 7

SEDIMENT EXTRACTION

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SEDIMENT EXTRACTION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample or extract.

Glassware (per sample)

250-mL tumbler/centrifuge bottle (amber, Boston round) with Teflon cap (Savillex, 24-mm)

500-mL 24/40-STJ Erlenmeyer flask with stopper

25-mL 19/22-STJ Kontes concentrator tube with stopper-

3-ball 24/40-STJ Snyder column

powder funnel (use more if needed)

Solvents/Reagents (per sample)

300 mL CH_2Cl_2 (not including washes)

50 g Na_2SO_4 (CH_2Cl_2-washed, dried, stored at 120°C, and cooled to room temp. in a desiccator just before, use)

hexane (as needed)

Standards Solutions

AH I-Std, PES I-Std, COP I-Std, AH spike, PES spike, and COP spike solutions

Other Materials and Apparatus

1 spatula per sample

modified rock tumbler (Model NF-1, Lortone Inc., 2856 NW Market St., Seattle, WA 98107; belt guard is removed)

centrifuge (to accommodate the tumbler/centrifuge bottles)

drying oven (120°C)

masking tape

desiccator

boiling chips.

1000-µL and 100-µL syringes

A. Equipment List (continued)

4 ea 2-mL GC vials

modified Kontes tube heater (Section 4A, page 25)

water bath

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

freezer (£ 20°C)

B. Sample Extraction

1. Decant the excess water from the top of the sediment. Stir the sediment to homogenize, and discard all pebbles, seaweed, wood, crabs, etc.
2. Using a spatula and powder funnel, weigh 10 + 0.5 g of sediment to the nearest 0.01 g into a tared bottle. Record the weight in the log book.
3. Set aside ca. 10 g of the homogenized sediment for the Dry Weight Determination (Section 9, page 65). Store the remaining sample in the freezer.
4. Centrifuge each sample bottle at £ 1500 rpm for 5 min. Decant and discard the H_20.
5. To each sediment sample add: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, and (d 100 µL of COP I-Std solution. Make certain that the solutions are placed into the CH_2Cl_2.
6. Prepare a spiked blank ("reagent spike") by adding to an empty bottle: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, (d) 100 µL of COP I-Std solution, (e) 100 µL of PES spike solution, (f) 100 µL of AH spike solution, and (g) 100 µL of COP spike solution.

B. Sample Extraction (continued)

7. Prepare a blank ("reagent blank") by adding to an empty bottle: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (C) 100 µL of PES I-Std solution, and (d) 100 µL of COP I-Std solution.

8. Prepare 2 AH/PES calibration-mixtures (for Section 11, step F.4, page 83) by adding to each of 2 vials: (a) 100 µL of AH spike solution, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, (d) 100 µL of PES spike solution, and (e) 600 µL hexane.

9. Prepare 2 COP calibration-mixtures (for Section 10, step G.3, page 76) by adding to each of 2 vials: (a) 800 µL of hexane, (b) 100 µL of COP I-Std solution, and (c) 100 µL of COP spike solution.

10. Add 50 g of Na_2SO_4 to each bottle in steps 5-7.

11. Screw each bottle cap on just tight enough to prevent leakage.

    CAUTION: Do not overtighten so as to deform the cap and cause leakage.

12. Tape the cap to the bottle (crosswise over the top with 2 strips of 1-inch-wide masking tape).

13. Manually shake each bottle until the contents are loose and free-flowing, then roll for 16 hr (i.e., overnight) on the tumbler at 100-250 rpm.

14. Remove the tape from each bottle, and centrifuge each bottle at < 1500 rpm for 5 min.

15. Decant each extract into a labeled flask.

16. Add 100 mL of CH_2Cl_2 to each sample, and repeat steps 11-14, except roll each bottle for 6 hr (i.e., during the day).

B. Sample Extraction (continued)

17. Decant the 2nd extract into the flask used in step 15.
18. Repeat step 16, except roll the bottle for 16 hr (i.e., overnight).
19. Add the 3rd extract from step 18 to the flask in step 17.

C. Concentration of Extract

1. Add 3-4 boiling chips to the flask containing the CH_2C1_2 extract from step B.18, and attach a Snyder column.
2. Concentrate the extract in a 60°C water bath to 10-15 mL, and transfer it to a labeled concentrator tube.
3. Wash the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube.
4. Repeat step 3 once.
5. Add a boiling chip to the tube, and using the tube heater, concentrate the extract to > 0.9 mL, < 1.0 mL.
6. Add 3 mL of hexane to the tube, and concentrate the extract to 2 mL on the tube heater.
7. Proceed to Silica Gel/Alumina Chromatography (Section 10, page 69).

SECTION 8

TISSUE EXTRACTION

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TISSUE EXTRACTION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample or extract.

Glassware (per sample)

100-mL centrifuge tube with Teflon-lined cap

500-mL 24/40-STJ Erlenmeyer flask with stopper

25-mL 19/22-STJ Kontes concentrator tube with stopper

3-ball 24/40-ST3 Snyder column

Solvents (per sample)

60 mL CH_2Cl_2 (not including washes)

20 g Na_2SO_4 (CH_2Cl_2-washed, dried, stored at 12O°C, and cooled to room temp. in a desiccator just before use)

hexane (as needed)

Standards Solutions

AH I-Std, PES I-Std, AH spike, and PES spike solutions

Other Materials and Apparatus

1 spatula per sample

Tekmar Tissumizer

drying oven (12O°C)

desiccator

centrifuge (to accommodate the 100-mL centrifuge tubes)

boiling chips

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

1000-µL and 100-µL syringes

2 ea 2-mL GC vials

A. Equipment List (continued)

water bath

modified Kontes tube heater (Section 4A, page 25)

Vortex Genie

freezer (£ -20°C)

Teflon sheeting to line centrifuge bottle caps

B. Sample Extraction

1. Using a spatula, and being careful to place the sample on the bottom and not the sides, weigh 3 t 0.5 g of sample to the nearest 0.01 g into the centrifuge tube, and set aside ca. 1 g for Dry Weight Determination (Section 9, page 65). Record the weight in the log book.
2. Store the remaining sample in the freezer.
3. To each tissue sample in a centrifuge tube add: (a) 25 mL of CH_2CL_2, (b) 20 µL of AH I-Std solution, and (c) 20 µl of PES I-Std solution. Make certain that the solutions are placed into the CH2C12.
4. Prepare a spiked blank ("reagent spike") by adding to a centrifuge tube containing 25 mL of CH_2Cl_2: (a) 20 µL of AH I-Std solution, (b) 20 µL of PES I-Std solution, (c) 20 µL of AH spike solution, and (d) 20 µL of PES spike solution.
5. Prepare a blank ("reagent blank") by adding to a centrifuge tube containing 25 mL of CH_2Cl_2: (a) 20 µL of AH I-Std solution, and (b) 20 µL of PES I-Std solution.
6. Prepare 2 calibration-mixtures (for Section 11, step G.5, page 83) by adding to each of 2 vials: (a) 1000 µL of hexane, (b) 20 µL of AH I-Std solution, (c) 20 µL of PES I-Std solution, (d) 20 µL of AH spike solution, and (e) 20 µL of PES spike solution.

B. Sample Extraction (continued)

7. Add 20 g of Na_2SO_4 to each tube in steps 3-5.
8. Macerate/extract the sample in the tube for 1 min with the Tissumizer at setting 100. Then continue at setting 50 for 2 min. Avoid spattering the tissue.
9. Centrifuge the sample for 5 min at £ 2000 rpm.
10. Decant the extract into a labeled flask.
11. Add 25 mL of CH_2Cl_2 to the tube.
12. Repeat steps 8-10 once.
13. Wash the Na_2SO_4/sample mass by adding 10 mL of CH_2Cl_2 to the tube, and mixing on the Vortex Genie for 5-10 seconds at setting 5-6.
14. Repeat steps 9-10 once.

C. Concentration of Extract

1. Add 3-4 boiling chips, and attach a Snyder column to the flask containing the CH_2Cl_2 extract from step B.14.
2. Concentrate the extract in a 60°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash the flask with 3-4 mL of CH_2C1_2, and add the washings to the tube.
4. Repeat step 3 once.
5. Add a boiling chip to the tube, and using the tube heater, concentrate the extract to > 0.9 mL, < 1.0 mL.
6. Add 3 mL of hexane to the tube, and concentrate the extract to 2 mL on the tube heater.
7. Proceed to Silica Gel/Alumina Chromatography (Section 10, page 69).

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SECTION 9

DRY WEIGHT DETERMINATION

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DRY WEIGHT DETERMINATION

A. Equipment List

analytical balances (requirements in steps B.4 and C.3)

spatula(s)

aluminum weighing pan(s)

aluminum foil, 12-inch width

drying oven (120°C)

desiccator

forceps

B. Sediment Procedure

1. Etch the sample number on the tab of the weighing pan.
2. Place up to 3 aluminum pans on l/2 of a 9-inch strip of aluminum foil. Fold aluminum foil over the weighing pan(s) to form an envelope. Close the envelope, but do not seal it, then place it in the drying oven overnight.
3. Cool the envelope containing the pan in a desiccator for 30 min.
4. Remove the pan from the envelope, and weigh the pan to the nearest 0.01 g. Record the pan weight as the Tare Weight in the log book.
5. Stir the sediment with a spatula to homogenize it, and discard pebbles, wood, roots, etc.
6. Add 10 ± 0.5 g of the sediment to the pan.
7. Record the weight to the nearest 0.01 g in the log book as the Wet Weight.

a. Return the weighing pan to the foil envelope, and close the envelope, but do not seal it.

B. Sediment Procedure (continued)

9. Dry the sample in the drying oven for 24 hr.
10. Remove the sample from the oven and cool it in the desiccator for 30 min.
11. Reweigh the sample, and record the dry weight to the nearest 0.01 g in the log book as the Dry Weight.

C. Tissue Procedure

1. Proceed as in steps B.1-B.4.
2. With a spatula spread ca. 0.5 g of tissue onto the pan.
3. Record the weight to the nearest 0.1 mg in the log book as the Wet Weight.
4. Proceed as in steps B.8-B.11, but record the weight to the nearest 0.1 mg.

D. Dry Weight Calculation

1. Calculate Dry Wt % as follows:

$$\text{Dry Wt \%} = \frac{\text{Dry Weight - Tare Weight}}{\text{Wet Weight - Tare Weight}} \times 100.$$

SECTION 10

SILICA GEL/ALUMINA CHROMATOGRAPHY OF SOLVENT EXTRACTS

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SILICA GEL/ALUMINA CHROMATOGRAPHY OF SOLVENT EXTRACTS

A. Equipment List - Note: CH_2C1_2-wash all glassware and materials contacting the extract or fractions.

Glassware (per sample)

19-mm ID chromatography column with reservoir

2 ea 250-mL beakers

50-mL TC graduated cylinder

2 ea* 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

2 ea* 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

2 ea* transfer pipets (Pasteur style) with rubber bulbs

2 ea* 3-ball 24/40-STJ Snyder columns

2-mL GC vial*

Reagents and Solvents (per sample)

20 g silica gel (heated to 700°C for 18 hr, stored at 170°C, and cooled to room temp. in a desiccator just before use)

10 g alumina (activated at 170°C for 18 hr, then cooled to room temp. in a desiccator just before use)

160/210 mL CH_2Cl_2 for tissue/sediment, resp. (not including washes)

25 mL 10% redistilled CH_3OH in CH_2Cl_2 (v:v), for sediment only

30 mL 20% redistilled CH_3OH in CH_2Cl_2 (v:v), for sediment only

50 mL pentane t the amount calibrated in Section 5 to elute the SA1 fraction

mL of 1:1 (v:v) CH_2Cl_2 pentane (the amount calibrated in Section to elute the SA2 fraction)

hexane, CH_2Cl_2 and redistil led CH_3OH, as needed

ca. 1 cc sand

For sediment: 7.5 cc copper (activated by covering it with conc. HCl and stirring with a glass rod, then allowing it to stand for 5 min, followed by washing twice with CH_3OH and then 3x with CH_2Cl_2)

* Add 1 ea for sediment samples

A. Equipment List (continued)

Standards Solutions

50 µL HMB GC I-Std solution per SA3 fraction

10 µL TCMX GC I-Std solution per SA1 fraction

Other Materials and Apparatus

desiccator

powder funnel

curved-stem funnel (curve glassblown)

glass rod

glass wool

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

boiling chips

modified Kontes tube heater (Section 4A, page 25)

water bath

Vortex Genie

100-µL and 10-µL syringes

B. Column Preparation

1. Prepare the columns just prior to use. On hot days elute more slowly to maintain the column integrity.
2. Fit a 19-mm ID column with a stopcock, add 100 mL of CH_2Cl_2 and a 5-15-mm glass-wool plug. Tamp the plug well to remove any bubbles.
3. Add the alumina to a beaker, and slowly add 20 mL of CH_2Cl_2. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 6.
4. Add the silica gel to a 2nd beaker. Slowly add 40 mL of CH_2Cl_2 to the beaker. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 7.

B. Column Preparation (continued)

5. Place a curved-stem funnel into the column reservoir so that the funnel tip hangs well off-center.
6. Swirl the beaker to resuspend the alumina from step 3, and pour the slurry into the column.
7. Wash the beaker with ca. 5 mL of CH_2Cl_2, and add the washings to the column. Repeat the wash twice.
8. After the particles settle, open the stopcock for 30 sec to allow the alumina to pack more tightly, then close the stopcock.
9. Add the silica gel from step 4 to the column, as in steps 6-7 for the alumina.
10. Place the beaker under the column tip, and open the stopcock. While the solvent still drains, add the sand through the powder funnel (for sediments: then add the copper). Drain to the packing top, thenclose the stopcock.
11. Add 50 mL of pentane to the column. Drain to the packing top, then close the stopcock. Discard the eluates collected thus far.

C. Chromatography of Extract

1. Wash the column tip with CH_2Cl_2, remove the waste beaker from beneath the column, and replace it with a cylinder labeled "SA1".
2. With a transfer pipet, carefully transfer the extract (in 2 mL of hexane) from Section 5, step C.6 (page 58) or Section 8, step C.6 (page 63) to the top of the packing. Drain to the packing top, then close the stopcock.
3. From the remaining pentane, wash down the tube that contained the extract from Section 7 or 8 with 0.5 mL, and add the washings to the top of the packing. Drain to the packing top, then close the stopcock.
4. Repeat step 3 once.

C. Chromatography of Extract (continued)

5. Wash down the tube with ca. 0.5 mL of 1:1 CH_2C1_2:pentane, and hold the washings in the tube for step 13.
6. From the remainder of the pentane, add ca. 2 mL to wash down the column wall. Drain to the packing top, then close the stopcock.
7. Repeat step 6 once.
8. Add the rest of the pentane, and continue eluting.
9. Collect _ mL of eluate (the amount calibrated in Section 5 for fraction SA1), then close the stopcock, and transfer the eluate to a flask labeled "SA1".
10. Wash down the cylinder with 3-4 mL of CH_2C1_2, and add the washings to the flask.
11. Repeat step 10 once, and set the flask aside for step E.1, page 75.
12. Replace the cylinder with a flask labeled "SA2". Drain to the packing top, then close the stopcock.
13. Add the washings from the tube (set aside in step 5) to the top of the packing. Drain to the packing top, then close the stopcock.
14. Wash down the tube with 0.5 mL of 1:1 CH_2Cl_2:pentane, and add the washings to the top of the packing. Drain to the packing top, then close the stopcock.
15. Add the remaining amount of 1:1 CH_2Cl_2:pentane to the column.
16. Partially open the stopcock, drain to the packing top at ca. 2 mL/min, and close the stopcock.
17. Set aside the SA2-labeled flask for step F.1, page 76.

D. For Sediment Only

1. Place a "waste" flask under the column, and add 50 mL of CH_2Cl_2 to the column. Drain to the packing top at ca. 2 mL/min, and close the stopcock.
2. Add 25 mL of 10% CH_3OH in CH_2Cl_2 to the column. Drain to the packing top at ca. 2 mL/min, and close the stopcock.
3. Discard the contents of the waste flask, and replace the flask with one labeled "SA3".
4. Add 30 mL of 20% CH_3OH in CH_2Cl_2 to the column. Elute all of the solvent into the SA3-labeled flask, and set it aside for step G.1, page 76.

E. Concentration of Fraction SA1

1. Add 3-4 boiling chips to the SA1-labeled flask from step C.ll, and attach a Snyder column.
2. Concentrate fraction SA1 in a 55°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube. Repeat this step once.
5. Add a boiling chip, and using the tube heater, concentrate the fraction to ^{3} 0.9 mL, < 1.0 mL.
6. Add 2 mL of hexane to the tube, and concentrate the fraction to > 0.9, < 1.0 mL.
7. Add 10 µL of TCMX GC I-Std solution to the tube, and mix for 2 sec on the Vortex Genie at setting B-10.

a. Transfer the concentrate into a GC vial, label it as "SA1", cap the vial, and store it in the freezer until needed.

F. Concentration of Fraction SA2

1. Concentrate the fraction in the SA2-labeled flask from step C.17 the same as for SA1 (steps E.1-E.6), except use a 60°C bath.
2. Add appropriate amounts of CH_3OH and CH_2Cl_2 to make £ 2.3 mL of a "6:4:3" solution (6 parts hexane, 4 parts CH_3OH, 3 parts CH_2Cl_2).
3. Proceed to Sephadex LH-20 Chromatography (Section 11, page 77).

G. Concentration of Fraction SA3 (Sediment Only)

1. Concentrate the fraction in the SA3-labeled flask from step D.4 the same as for SA1 (steps E.1-E.7), except use a 75° C bath, add 7 mL of hexane, and add 50 µL of HMB GC I-Std solution instead of the TCMX GC I-Std solution.
2. Transfer the concentrate into a GC vial, label it as "SA3", and cap the vial.
3. Add 50 µL of HMB GC I-Std solution to the COP calibration-mixture vials from Section 7, step B.9 (page 57).
4. Proceed to GC Analysis (Section 12, page 85), and analyze for coprostanol.

SECTION 11

6:4:3 SEPHADEX LH-20 CHROMATOGRAPHY

AHs and Chlorinated HCs from Fraction SA2

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6:4:3 SEPHADEX LH-20 CHROMATOGRAPHY

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting extract or fractions.

Glassware (per sample)

50-mL TC graduated cylinder

100-mL TC graduated cylinder

2 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

2 ea 3-ball 24/40-STJ Snyder columns

2 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials (substitute 1 conical vial for tissue extract)

Solvents (per sample)

200 mL 6:4:3 cyclohexane:CH_3OH:CH_2Cl_2 solution

2 mL redistilled CH_3OH

GC Calibration Standards (per SA2-L2 fraction)

HMB GC I-Std solution: 50 µL (sediment); 10 µL (tissue)

TCMX GC I-Std solution: 50 µL (sediment); 10 µL (tissue)

Other Materials and Apparatus

aluminum foil

activated copper (Section 10A, page 71)

Vortex Genie

calibrated Sephadex LH-20 columns (Section 6, page 45)

boiling chips

water bath

A. Equipment List (continued)

modified Kontes tube heater (Section 4A, page 25)

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

freezer (< -20°C)

2 ea syringes: 100-µL (sediment); 10-µL (tissue)

B. Special Notes

1. The 6:4:3 solvent must be tested before using to assure freedom from interfering contaminants (Section 4B, page 29).
2. The extract must be dissolved in the solvent (no layers), with the total volume < 2.3 mL.
3. The fraction volumes are dependent on the column calibration. Occasionally check the column calibration with the "calibration-extract" (Section 6, Part A, page 48).
4. Extreme care must be used when removing or adding solvent or sample to the column to avoid disturbing the column packing.
5. During column storage, if the solvent in the reservoir separates into 2 phases, remove it and replace it with > 80 mL of fresh 6:4:3 solvent, then elute 50 mL.

c. Chromatography

1. Remove the excess solvent from the top of the column using a transfer pipet.
2. Add 10 mL of the 6:4:3 solvent to the column. Drain to the packing top, and close the stopcock. Discard the eluate.
3. Wash the column tip with CH_2Cl_2, and place the 50-mL cylinder labeled "SA2-L1", under the column.
4. Using a transfer pipet, carefully apply the 2-mL extract from Section 10, step F.2 (page 76) to the column. Use a circular motion

C. Chromatography (continued)

to dispense the sample immediately above the packing, dripping it slowly down the column wall so as not to disturb the packing.

5. Drain to the packing top, and close the stopcock.
6. Wash down the tube with 0.5 mL of the solvent, and apply the washings to the column. Drain to the packing top, and close the stopcock.
7. Repeat step 6 once.
8. Wash down the column wall with ca. 3 mL of the solvent, applied above the base of the reservoir. Drain to the packing top, and close the stopcock.
9. Repeat step 8 once.
10. Carefully add 150 mL of the solvent to the column (add more as needed) without disturbing the packing.
11. Collect mL of eluate (the amount calibrated in Section 6 for fraction SA2-L2) in the 50-mL cylinder labeled "SA2-L1". Close the stopcock, and transfer the eluate to a flask labeled "SA2-L1".
12. Wash down the cylinder with 3-4 mL of CH_2Cl_2, and add the washings to the flask.
13. Repeat step 12 once, and set the flask aside for step D.1.
14. Replace the 50-mL cylinder with the 100-mL cylinder labeled "SA2-L2." Open the stopcock, and collect mL of eluate (the amount calibrated in Section 6 for fraction SA2-L2). Close the stopcock, and transfer the eluate to a flask labeled "SA2-L2". Repeat steps 12 and 13, except set the flask aside for step E.1.
15. Replace the 100-mL cylinder with a "waste" cylinder, and elute 50 mL of solvent to flush the column. Discard this eluate.

C. Chromatography (continued)

16. The column is now ready for the next sample.

Note: If the column is to be stored for awhile, maintain 30-50 mL of the solvent in the column reservoir, and cover the top with aluminum foil to minimize evaporation. Remove the solvent if it separates into 2 phases, add > 80 mL fresh 6:4:3 solvent, and elute 50 mL.

D. Concentration of Fraction SA2-L1

1. Add 3-4 boiling chips to the flask from step C.13, and attach a Snyder column.
2. Concentrate the fraction in a 75°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash the flask with 3-4 mL of CH_2C1_2, and add the washings to the tube. Repeat this once.
4. Add 1 mL of CH_3OH and a boiling chip to the tube, and using the tube heater, concentrate the fraction to ³ O.9 mL, < 1.0 mL.
5. Add 7 mL of hexane to the tube, and concentrate the fraction to ³ 0.9 mL, < 1.0 mL.
6. Transfer the fraction to a GC vial, cap the vial, label it, and store it in the freezer until needed.

E. Concentration of Fraction SA2-L2

1. Concentrate fraction SA2-L2 from step C.14 in the same manner as in steps D.1-D.5 above.
2. Proceed to step F.1 for sediment or step G.1 for tissue.

F. Fraction SA2-L2 from Sediment

1. Add 50 µL of HMB GC I-Std solution and 50 µL of TCMX GC I-Std solution to the tube, using the 100-µL syringes. Mix on the Vortex Genie for 2 sec at setting 8-10.

F. Fraction SA2-L2 from Sediment (continued)

2. Add a few grains of activated copper (Subsection 4A, page 25) until no further discoloring occurs.
3. Transfer equal amounts of the fraction to 2 GC vials, cap the vials, and label them.
4. Add 50 µL of HMB GC I-Std solution to the AH/PES calibration-mixture vials from Section 7, step B.8 (page 57), using the 100-µL -syringes. Similarly, add 50 µL of TCMX GC I-Std solution to these vials.
5. Add "R" to the label of one of the vials from step 3 and from step 4, and store them in the freezer as reserves. Proceed with the other vials from steps 3 and 4 to GC analysis (Section 12, page 85).

G. Fraction SA2-L2 from Tissue

1. Add 10 µL of HMB GC I-Std solution and 10 µL of TCMX GC I-Std solution to the tube (from step E.2, page 82), using the 10 µL syringes.
2. Mix on the Vortex Genie for 2 sec at setting 8-10.
3. Transfer equal amounts of the fraction to 2 GC vials, cap the vials, and label them.
4. Add "R" to the label of one vial, and store it in the freezer as a reserve GC sample. Set aside the other vial for step 7.
5. Add 10 µL of HMB GC I-Std solution and 10 µL of TCMX GC I-Std solution to the calibration-mixture vials from Section 8, step B.6 (page 62), using the 10-µL syringes. Mix on the Vortex Genie for 2 sec at setting 8-10, and recap the vials.

G. Fraction SA2-L2 from Tissue (continued)

6. Add "R" to the label of one vial from step 5, and store it in the freezer as a reserve GC sample. Proceed with the other vial to GC Analysis (Section l2, page 85).

7. Using a pipet, transfer a portion of tissue fraction SA2-L2 from step 4 to a conical GC vial. Place this vial under a gentle stream of nitrogen gas (piped through only CH_2Cl_2-washed Teflon, stainless-steel,or glass tubing), and slowly evaporate part of the solvent.

8. Repeat step 7 until the entire contents of the larger GC vial has been transferred to the conical GC vial. The volume of the concentrated fraction should be ca. 0.1 mL.

9. Cap the vial, label it, and proceed to GC Analysis (Section 12, page 85).

SECTION 12

GC ANALYSIS

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GC ANALYSIS

A. Equipment List - Note: Wash the Autosampler syringe thoroughly with CH_2C1_2 before using.

Gas Chromatograph (GC), Hewlett-Packard model 5880A, including:

capillary column inlet system, in the No. 2 position

automatic sample injector

cartridge tape unit

flame-ionization detector (FID), in the No. 1 position

electron-capture detector (ECD), in the No. 2 position

modifications: Two O-rings are placed around the injector insert instead of 1. An, O-ring is installed beneath the septum. A slot is cut in 2 ea 1 x l-inch, 1/32-inch-thick aluminum plates so that they may be inserted from opposite sides around the injection port, just above the gas lines, and between the septum retainer assembly and the insert retainer assembly. A 1/16-inch tube is installed to blow compressed air gently onto the cooling fins.

Standards Solutions

AH GC-calibration check solution

PES GC-calibration check solution

COP GC-calibration check solution

Gas Cylinders

air, Ohio breathing air, CGA Grade E (or equivalent)

argon/methane, 95/5 (v/v)

helium, grade 4.5 (purified, ^{3} 99.995%)

hydrogen, grade 5 (ultra pure, ^{3} 99.999%)

nitrogen, grade 4.5 (purified, ^{3} 99.995%)

molecular sieve traps (1 for each gas cylinder) - Hydro-Purge model ASC-1, Coast Engineering Laboratory, Gardena, CA

regulators (1 for each gas cylinder), 2-stage

Solvents

CH_2Cl_2 and washing CH_3OH, as needed

A. Equipment List (continued)

Other Materials and Apparatus

GC column, J & W Scientific Inc., fused silica, DB-5, 30-m

diamond-tip etcher

2 ea ferrules, J & W Scientific Inc., 0.4-mm graphite, part no. 500-2004

jeweler's loupe, 10x

Wite-Out, typewriter correction fluid, Wite-Out Products Inc. Beltsville, MD

leak detector, Snoop, Nupro Co.

septum, Alltech Associates, 3/8-inch, blue, stock no. 6514

3 ea O-rings, Viton 0.208-inch ID, Parker Seal Co.

brush for cleaning fused-silica liner

glass-wool (as necessary)

10 µL syringe, Hamilton model 1701

2-mL GC vials, Varian, part no. 96-000099-00

100-µL conical GC vials, Wheaton, part no. 986281

flowmeter(s) suitable for all gases used

soap

1/8-inch OD copper tubing

Swagelok adapters (as necessary)

1/8-inch Swagelok tee

1/8-inch Swagelok connectors (as necessary)

B. Column Installation for FID Operation

1. Place a new column on the rack holder in the GC oven.
2. Unwind the ends so that one faces the injector inlet and the other faces the detector outlet.
3. Slide the column nut over the inlet end of the column.

B. Column Installation for FID Operation (continued)

4. Using the diamond-tip etcher, score the column lightly about 1 cm from the column end.
5. Snap off the column tip at the etched point.
6. Slide the ferrule over the inlet end of the column.
7. Etch the column again ca. 2 mm below the previously cut end.
8. Snap off the 2 mm above the etched point.
9. Examine the end with the jewelers loupe; if it is not smooth (clean cut) and perpendicular to the column sides, repeat steps 7 through 9.
10. Slide the column nut up the column until only 35 mm of column extends beyond the base of the ferrule.
11. With Wite-Out, place a white mark on the column even with the base of the column nut.
12. Slide the column up into the injector, and tighten the nut by hand until the column is held lightly in place.
13. Adjust the column so that the white mark is again even with the base of the nut, then tighten the nut just sufficiently that no gas escapes when tested with a leak detector.
14. Tighten nut an additional 1/4 turn.
15. Repeat steps 3 through 9 on the detector end of the column.
16. Slide the column nut up the column until 57 mm of the column extends beyond the ferrule.
17. Repeat step 11.
18. Slide the column up into the FID, and tighten the nut by hand until the column is held lightly in place.
19. Repeat steps 13 and 14 only.

C. Column Installation for ECD Operation

1. Repeat steps B.1-B.19.
2. Slide the column nut up the column until only 47 mm of the column extends beyond the ferrule.
3. Repeat steps B.17-B.19, except use the ECD.

D. Injector Maintenance

1. Cool the injector-and the oven to near room temp, then loosen the column nut at the injector block.
2. Disconnect the air from the auto-sampler, and remove the sample tray.
3. Make sure that the auto-sampler door is closed and the carrier gas pressure is released, then tilt the auto-sampler back.
4. If the gases are not already installed, proceed to Part E first. Otherwise, turn off the cooling air and the carrier gas ("carrier C/D" valve).
5. Remove the septum retaining nut. Discard the septum if it is worn. Check the O-ring, and replace it if it is worn or cracked.
6. Unscrew the lower injector cover, and withdraw the fused-silica liner.
7. Remove the O-rings, and set them aside for step 12.
8. Using a small stiff brush, wash inside and outside of the liner with soap and water.
9. Flush the liner thoroughly with water.
10. Hold the liner with a clamp, and wash it with CH_3OH and then CH_2Cl_2.
11. After the liner has dried, use a pair of small forceps and a small glass rod to place a 5-mm, lightly-packed, glass-wool plug into the liner, and push it 35 mm below the septum end of the liner.
12. Check the O-rings that fit on the injector insert, and replace any that are worn or cracked.

D. Injector Maintenance (continued)

13. Slide the O-rings onto the injector liner, and place the liner back into the injector, being careful to slide the column into the liner.
14. Reattach the injector cover.
15. Replace the O-ring on top of the injector cover.
16. Replace the septum on top of the O-ring in step 15.
17. Screw on the septum retaining nut, and adjust the cooling fins.
18. Turn on the cooling air, and retighten the column nut at the injector block.
19. Adjust the carrier gas pressure to 20 psi.
20. Return the injector temperature to 300°C and the oven temperature to 180°C.

E. Installation of Gases for FID

1. Attach a 2-stage regulator to a full nitrogen cylinder.
2. Connect a molecular-sieve trap to the nitrogen regulator.
3. Connect the trap to the "Aux 2" (make-up gas connector) gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
4. Open the nitrogen-cylinder valve, and adjust the output pressure of the regulator attached to the nitrogen cylinder to read 50 psi.
5. Check all connections of the nitrogen delivery system with leak detector, and tighten or replace any that leak.
6. Adjust the Aux 2 pressure to read 30 psi on the gauge on the front panel of the GC.
7. Attach a 2-stage regulator to a full hydrogen cylinder.
8. Connect a molecular-sieve trap to the hydrogen regulator.
9. Connect the trap to the hydrogen gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.

E. Installation of Gases for FID (continued)

10. Open the gas cylinder valve, and adjust the output pressure of the regulator attached to the hydrogen cylinder to read 55 psi.
11. Repeat step 5 with the hydrogen delivery system.
12. Adjust the hydrogen pressure to 30 psi on the gauge on the front panel of the GC.
13. Attach a 2-stage regulator to a full helium cylinder.
14. Connect a molecular-sieve trap to the helium regulator.
15. Connect the trap to the "carrier C/D" gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
16. Open the helium-cylinder valve, and adjust the output pressure of the regulator attached to the helium cylinder to read 40 psi.
17. Repeat step 5 with the helium delivery system.
18. Adjust the carrier gas pressure to read 20 psi on the "carrier C/D" gauge on the front panel of the GC.
19. Attach a 2-stage regulator to a full breathing-air cylinder.
20. Connect a molecular-sieve trap to the air regulator.
21. Connect a 1/8-inch Swagelok tee to the outlet of the trap. Connect one end to the auto-sampler and the other end to the "air" gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
22. Open the air-cylinder valve, and adjust the output pressure of the regulator attached to the air cylinder to 75 psi.
23. Repeat step 5 with the air delivery system.
24. Adjust the air pressure to read 30 psi on the gauge on the front panel of the GC.
25. Attach the high-flow line from the gas flowmeter to the split vent.
26. Adjust the injector split vent flow using the C flow valve to 40 mL/min.

E. Installation of Gases for FID (continued)

27. Attach the low-flow line from the gas flowmeter to the septum purge vent.
28. Adjust the septum purge flow using the septum purge valve to ca. 10 mL/min.
29. Make sure that the injector split vent flow is still 40 mL/min.
30. Detach both flowmeter lines.

F, Installation of Gases for ECD

1. Attach a 2-stage regulator to a full argon/methane cylinder.
2. Connect a molecular-sieve trap to the argon/methane regulator.
3. Connect the trap to the "Aux 2" (make-up gas) gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
4. Open the argon/CH_4-gas cylinder valve, and adjust the output pressure of the regulator attached to the cylinder to 60 psi.
5. Adjust the "Aux 2" pressure to read 30 psi on the gauge on the front panel of the GC.
6. Check all connections with leak detector, and tighten or replace any that leak.
7. Repeat steps E.13-E.23 and E.25-E.30 (pages 92 and 93), making certain that the air valve on the front of the GC remains turned off.

G. Entering and Storing Program "ROUTINE" - The GC Program (explanatory notes on right margin in parentheses)

1. Press the CLEAR ENTRY button on terminal 1.
2. Press the ENTER button on terminal 1.
3. Type the following lines (letters will appear capitalized in the GC printout), and press the RETURN button after each line:

G. Entering and Storing Program "ROUTINE" (continued)

```
 10 option base 1
 20 rem overnight sample runs
 25 gosub 1300                          (set up Autosampler information)
 30 dim s(25)
 40 for i=l to 25
 50 s(i)=0
 60 next i
 70 input "total number of samples to run",n      (enter how many GC vials
                                                   are to be analyzed)
 80 If n<26 then 110
 90 print "maximum of 25 samples allowed"
100 got0 70
110 input "enter starting bottle number",b
120 for i=1 to n                                  (store vial nos. for
                                                   each sample)
130 s(i)=b
135  b=b+2
140 print "enter sample name for bottle #";s(i)      (enter sample name)
145 if i>14 then 151
150 on i goto 160,180,200,220,240,260,280,300,320,340,360,380,400,420
151 on i-14 goto 440,460,480,500,520,540,560,580,600,620,640
152 print "on goto error - line 150-151"
153 goto 2000
160 input a$
170 goto 660
180 input b$
190 goto 660
200 input c$
```

G. Entering and Storing Program "ROUTINE" (continued)

```
210 goto 660
220 input d$
230 goto 660
240 input e$
250 goto 660
260 input f$
270 goto 660
280 input g$
290 got0 660
300 input h$
310 goto 660
320 input i$
330 goto 660
340 input j$
350 goto 660
360 input k$
370 goto 660
380 input l$
390 goto 660
400 input m$
410 goto 660
420 input n$
430 goto 660
440 input o$
450 goto 660
460 input p$
```

G. Entering and Storing Program "ROUTINE" (continued)

```
470 goto 660
480 input q$
490 goto 660
500 input r$
510 goto 660
520 input s$
530 goto 660
540 input t$
550 goto 660
560 input u$
570 goto 660
580 input v$
590 goto 660
600 input w$
610 goto 660
620 input x$
630 goto 660
640 input y$
660 next i
670 input "which analysis file to use",z$          (get GC conditions
                                                    from Analysis File
680 execute x, "get analysis """&z$""" device# 6"          on tape)
685 if x<>0 then 2000
690 For i=1 to n                                   (print sample name
                                                      on chart)
695 wait
696 list
700 print using 710; "sample:"
```

G. Entering and Storing Program "ROUTINE" (continued)

```
710 image #,10/,5x,8a
720 image x,50a,2/
725 if i>14 then 731
730 on i goto 740,760,780,800,820,840,860,880,900,920,940,960,980,1000
731 on i-14 goto 1020, 1040,1060,1080,1100,1120,1140,1160,1180,1200
732 print "on goto error in line 730"
733 got0 2000
740 print using 720;a$
750 goto 1230
760 print using 720;b$
770 goto 1230
780 print using 72O;c$
790 goto 1230
800 print using 720;d$
810 goto 1230
820 print using 720;e$
830 goto 1230
840 print using 720;f$
850 goto 1230
860 print using 72O;g$
870 goto 1230
880 print using 720;h$
890 goto 1230
900 print using 72O;i$
910 goto 1230
920 print using 72O;j$
```

G. Entering and Storing Program "ROUTINE" (continued)

```
 930 goto 1230
 940 print using 720;k$
 950 goto 1230
 960 print using 720;l$
 970 goto 1230
 980 print using 720;m$
 990 goto 1230
1000 print using 720;n$
1010 goto 1230
1020 print using 720;o$
1030 goto 1230
1040 print using 720;p$
1050 goto 1230
1060 print using 720;q$
1070 goto 1230
1080 print using 720;r$
1090 goto 1230
1100 print using 720;s$
1110 goto 1230
1120 print using 720;t$
1130 goto 1230
1140 print using 720;u$
1150 goto 1230
1160 print using 720;v$
1170 goto 1230
1180 print using 720;w$
```

G. Entering and Storing Program "ROUTINE" (continued)

```
1190 goto 1230
1200 print using 72O;x$
1210 goto 1230
1220 print using 72O;y$
1230 rem
1240 valve 6 on                                  (close inlet-purge valve;
                                                    inject next GC sample)
1250 execute x, "edit auto seq 8, "&val$(s(i)+l)
1260 if x<>0 then 2000
1270 start auto seq s(i),s(i)
1280 next i
1290 oven temp initial  value 180
1300 execute x, "edit auto seq 1,2"
1310 if x<>0 then 2000
1320 execute x, "edit auto seq 2,O"
1330 if x<>0 then 2000
1340 execute x "edit auto seq 3,5"
1350 if x<>0 then 2000
1360 execute x, "edit auto seq 4,1"
1370 if x<>0 then 2000
1380 execute x, "edit auto seq 5,1"
1390 if x<>0 then 2000
1400 execute x, "edit auto seq 9,lO"
1410 if x<>0 then 2000
1420 return
2000 end
save prgm "routine" device# 6.
```

H. Entering/Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-l]

Note: The options for GC analysis files are:
HEXANE (for 1 mL GC/FID samples);
HEXANE CON-l (for 0.1 mL GC/FID samples), follow brackets [ ];
HEXANE EC (for 1-mL GC/ECD samples), follow parentheses ( ).

1. Press the CLEAR ENTRY button on terminal 1.

2. Press the ENTER button on terminal 1.

3. Type the following lines (letter will appear capitalized in the GC printout), and press the RETURN button after each line.

oven temp limit 320

oven temp 50

oven temp on

det 1 temp limit 325 [det 2 temp limit 325]

det 1 temp 320 [det 2 temp 320]

det 1 temp on [det 2 temp on]

inj 2 temp limit 320

inj 2 temp 300

inj 2 temp on

detector b on [detector c on]

delete run tbl

run tbl on

run tbl annotation on

run time 0.50 valve 6 off

run time 80.00 stop

signal B device# 12 [signal c device# 12]

signal on device# 12

stop plot device# 12

chart speed 0.70 device# 12

H. Entering/Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-1) (continued)

attn 2 - 2 device# 12 [attn 2 - 8 device #12] (attn 2 - -1 device #12)

%offset 10 device# 12

zero on device# 12

intg signal B [intg signal c]

sync off

run time annotation on

oven temp equib time 1.00

delete oven temp

oven temp initial time 3.00

oven temp 1 prgm rate 4.00

oven temp 1 final value 300

oven temp 1 final time 10.00

oven temp annotation on

valve 6 off

peak width 0.04

threshold -3 [threshold 6]

report on device# 0

report on

report annotation on

delete report tbl

report time 0.00 reject le+16

report time 1.00 bl mode 0

report time 5.00 reject 0.1 [report time 5.00 reject 100]

area%

delete calib

H. Entering and Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-l) (continued)

edit calib 0,l

edit calib -1,5

edit calib -2,5

edit calib -3,0

edit calib -4,""

edit calib -5,0

save analysis "hexane" device# 6 [save analysis "hexane ec" device# 6]
(save analysis "hexane con-l" device# 6)

I. Verification of Stable GC Performance - Note: The options for analysis files:
HEXANE (for GC/FID check);
HEXANE EC (for GC/ECD check).

1. Place 6 vials containing the desired GC-calibration check solution in the first 6 odd-numbered slots of the autosampler.
2. Place hexane-filled wash-vials in the even-numbered slots following each GC-calibration check solution vial.
3. Press the GET and PRGM buttons, then type "routine" (including the quotation marks; letters will appear capitalized).
4. Press the following buttons: DEVICE# 6 and RETURN. After typing the correct response to each question that is asked, press the RETURN button. For analysis file option, see Note above.
5. Use the 6th GC injection to calculate the analyte peaks in the previous calibration runs as if they were unknowns (Part K, page 104).
6. The GC is operating properly if the deviation between calibrations is < 5% for any analyte standard peak in calibrations #3-#6. If this criterion is not met, troubleshoot/adjust/repair the GC instrument, and repeat steps 1-5 until the criterion is met.

J. Analyses of Sample Extract Fraction Concentrates (GC Samples)

1. If there are n GC samples to be analyzed, let c be the next larger integer than [n/4 + 3].
2. Take 1 of the 2 calibration-mixture vials prepared with the set and split it into c subsamples in conical GC vials. Cap these calibration-mixture conical (CMC) vials and use them as the calibration standards in the analyses.
3. Load the sample vials into the auto-sampler tray, putting a CMC vial in the 1st 3 odd-numbered slots and the GC sample vials in the remaining odd-numbered slots, making certain that every 5th odd slot holds a CMC vial and that the last slot is taken by a GC-calibration check solution.
4. Place hexane-filled wash-vials in the even numbered slots following each sample vial or CMC vial.

Note: It is very important that the GC vial contents be analyzed in a consistent manner. For example, the reproducibility of replicate GC analysis data depends greatly on the duration of the 50° C resting period between analytical runs. To test the reproducibility, perform 3 calibrations at the beginning of the GC sample set, followed by another calibration every 5th GC injection. Repeatability can be checked by calculating the GC-calibration check solution as if it were a GC unknown. The 3rd or 4th calibration is commonly used to calculate the percent recoveries of the other calibrations. A difference > 5% between calibrations indicates a problem with the GC system (e.g., a leaking septum, a loose ferrule, a worn out or dirty column, etc.). Such problems should be rectified before proceeding with analyses of sample extracts. To check the integrity of the calibration solution in the CMC vial, analyze the last CMC vial as an unknown with the GC-calibration check solution used as the calibration standard. Again, a difference > 5% between the CMC vial and the GC-calibration check solution indicates a problem with the calibration solution in the CMC vial. The latter must then be checked against the alternate calibration-mixture vial to determine precisely what the problem is.

J. Analyses of Sample Extract Fraction Concentrates (GC Samples) (continued)

5. Press the GET and PRGM buttons, then type "routine" (including the quotation marks; letters will appear capitalized).

6. Press the following buttons: DEVICE# 6 and RETURN. After typing the correct response to each question that is asked, press the RETURN button. For analysis file option, see Note in Part H.

K. Analyte and I-Std Calculations

Identify the analyte peaks in the chromatograms of the sample extracts by comparing them with the analyte retention times obtained from the chromatograms of the calibration standards. Samples analyzed on the ECD that have peaks in addition to those peaks in the standards, i.e., aroclor (PCB) mixtures, need to be analyzed by GC-MS to identify the component chemicals and to verify the analytes previously identified by retention time comparisons. The GC-MS chromatograms are then used to label the peaks in the GC/ECD chromatogram.

The I-Std(s) added to the sample at the beginning of the extraction (e.g., naphthalene-d_8, acenaphthene-d_{10}, and perylene-d_{12}); are used to adjust for analyte losses during sample workup. Equation 12-1 (page 105) is used for calculating the analyte concentration in the sample on a dry weight basis.

Equation 12-2 (page 106) is used for calculating the percent recovery of the I-Std(s), and it involves the use of a GC I-Std which is added to the fractionated, concentrated extract in the GC vial (e.g., HMB and/or TCMX). If the I-Std recovery is < 50%, reanalysis of the unused portion of the sample (or the reserve vial) is indicated.

K. Analyte and I-Std Calculations (continued)

Equation 12-1, calculation of the concentration of an analyte in a marine sediment or tissue sample, dry weight basis:

$$\frac{\text{ng of analyte}}{\text{g sample, dry wt}} = \frac{R_1 \times R_2}{R_3} \times \frac{\text{ng I-Std added to sample}}{\text{sample wt}} \times \frac{100}{\%\text{ dry wt}}$$

where

$$R_1 = \frac{\text{analyte peak area in GC analysis}}{\text{I-Std peak area in GC analysis}}$$

$$R_2 = \frac{\text{analyte concentration in- calibrating solution (ng/µL)}}{\text{I-Std concentration in calibrating solution (ng/µL)}}\text{, and}$$

$$R_3 = \frac{\text{analyte peak area in GC calibration}}{\text{I-Std peak area in GC calibration}}$$

J. Analyte and I-Std Calculations (continued)

Equation 12-2, calculation of percent (%) recovery of internal standard (I-Std):

$$\% \text{ recovery of I-Std} = \frac{R_1 \times R_2}{R_3} \times \frac{\text{ng GC I-Std added to GC vial}}{\text{ng I-Std added to marine sample}} \times 100 ,$$

where

$$RI = \frac{\text{I-Std peak area in GC analysis}}{\text{GC I-Std peak area in GC analysis}} ,$$

$$R_2 = \frac{\text{I-Std concentration in calibrating solution (ng/µL)}}{\text{GC I-Std concentration in calibrating solution (ng/µL)}} , \text{ and}$$

$$R_3 = \frac{\text{I-Std peak area in GC calibration}}{\text{GC I-Std peak area in GC calibration}} ,$$

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