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WESTON SOLUTIONS

APPENDIX E

STANDARD OPERATING PROCEDURES – INVESTIGATORS

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BIOMONITORING OF ENVIRONMENTAL STATUS AND TRENDS (BEST) PROGRAM: FIELD PROCEDURES FOR ASSESSING THE EXPOSURE OF FISH TO ENVIRONMENTAL CONTAMINANTS [USGS-TILLITT]

BIOMONITORING OF ENVIRONMENTAL STATUS AND TRENDS (BEST) PROGRAM: FIELD PROCEDURES FOR ASSESSING THE EXPOSURE OF FISH TO ENVIRONMENTAL CONTAMINANTS

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1.0 INTRODUCTION

This guide describes field procedures to be used in the conduct of investigations designed to monitor and assess the exposure of fish to environmental contaminants. It describes how to observe and handle freshly captured fish, and how to collect, store, and ship the tissues and fluids necessary for a suite of monitoring methods being evaluated by the U.S. Geological Survey (USGS) Biomonitoring of Environmental Status and Trends (BEST) Program. Although the guide was developed for specific pilot studies being conducted by the BEST Program (Schmitt et al. 1995; Bartish et al. 1997), it and the specific chemical and biological methods to which it refers are potentially adaptable to a wide variety of situations and habitats. It is not a guidance document for selecting methods, nor does it contain the information necessary to interpret the results of studies based on the use of the guide and the methods it supports. The rationale for selecting this particular suite of methods, as well as information on the interpretation of findings, is presented elsewhere (NBS 1995; Schmitt et al. in press--methods summary).

The procedures described here are primarily post-collection; although the guide includes information on preparing for and organizing a study and suggests methods for documenting the date, time, and location of collection, most of the actual procedures begin after fish have been collected. Procedures are specified for making observations and collecting information, tissues, and fluids; preserving, wrapping and packaging samples; numbering samples and recording data; and storing and shipping samples and records. The guide also provides instructions for documenting the type of equipment used to collect the fish, the duration of the fishing effort, the exact location of the collection site, and the identity and qualifications of the personnel involved in the collection and processing of specimens and samples. A list of suggested equipment and supplies is also provided. To the extent possible, the procedures conform to Good Laboratory Practices (U.S. Environmental Protection Agency 1989); however, quality assurance and quality control issues are beyond the scope of this document. Depending on the purpose of the study, it may be necessary to develop additional project-specific protocols and SOP(s) conforming to this guide, as well as a quality assurance plan. This guide and its sample data sheets may be photocopied or otherwise reproduced on waterproof paper for field use.

1.1 Background

Field portions of BEST pilot projects using the methods described in this guide were conducted in 1995 (Schmitt et al. 1995) and 1997 (Bartish et al. 1997). In these projects, fish were collected at National Contaminant Biomonitoring Program (NCBP) stations (Schmitt et al. in press--NCBP paper) and from other USGS sites in the Mississippi, Columbia, and Rio Grande basins. The U.S. Fish and Wildlife Service (FWS) had periodically collected freshwater fish at the national network of NCBP sites from 1967 through 1986 (Schmitt and Bunck 1995; Schmitt et al. in press). The NCBP fish were analyzed for persistent organochlorine and elemental contaminants. The overall objectives of the 1995 and 1997 projects were therefore (1) to test the feasibility of implementing biological monitoring methods recommended for use in the BEST program (Schmitt et al. in press--methods summary); and (2) to update information on the distribution and

concentrations of organochlorine and elemental contaminants in selected large U.S. rivers.

In addition to the collection and analysis of composite whole fish for elemental and organic chemical residues following NCBP protocol (Schmitt et al. in press--NCBP paper), the following methods were incorporated into the pilot projects: (1) the H4IIE rat hepatoma cell bioassay, a sensitive *in vitro* method for documenting the exposure of organisms to planar polyhalogenated hydrocarbons (Tillitt et al. 1991); (2) hepatic ethoxyresorufin-O-deethylase (EROD) activity, an indicator of exposure to polyhalogenated and polycyclic aromatic hydrocarbons (Pohl and Fouts 1990); (3) condition indices and other indicators of general fish health and well-being, including gross changes and the quantitative field health assessment described in this guide (Goede 1989; Adams 1990; Goede and Barton 1990; Adams et al. 1993); and (4) histopathological examination of selected tissues and structures (Hinton et al. 1992; Hinton 1993). The composite fish carcass samples were also analyzed for stable isotopes of nitrogen ($\delta^{15}N$), a potential indicator of trophic position and nitrogen inputs (Cabana and Rasmussen 1996). Because of continuing interest in the effects of chemicals on reproduction (e.g., Colborn 1991; Colborn et al. 1993; Guillette et al. 1994), biomarkers diagnostic of reproductive health and endocrine modulation were also evaluated. These included plasma vitellogenin (Folmar et al. 1996) and sex steroid hormone concentrations (Guillette et al. 1994; Gilliom et al. 1994; Goodbred et al. 1997), and gonadal histopathology (Goodbred et al. 1997). Contaminants can also suppress immune system function (e.g., Matthews et al. 1990; Hutchinson and Simmonds 1994). Consequently, two immune system indicators -- splenic macrophage aggregates (Blazer et al. 1994, 1997) and plasma lysozyme activity (Blazer et al. 1994) -- were included.

1.2 Development of this Document

This guide originated as a Standard Operating Procedure (SOP) developed by the USGS Columbia Environmental Research Center (CERC) for the 1995 and 1997 BEST pilot studies of environmental contaminants and their effects on fish in the Mississippi, Columbia, and Rio Grande basins (Schmitt et al. 1995; Bartish et al. 1997). Following field portions of each study, the BEST program sponsored workshops at which participants (program staff, investigators, cooperators, and partners) identified specific procedures, documentation, and materials that could be improved to further expedite the processing of specimens and samples, which were incorporated into subsequent revisions of the SOP. This guide also incorporates information and procedures from numerous other sources, including other CERC SOPs; study plans and reports from NAWQA investigations (Goodbred et al. 1994, 1997); and books, manuals, and reports from other studies (Lagler et al. 1962; Smith 1973; Hunn 1988; Meyer and Barclay 1990; Baker et al. 1997). The health assessments based on field observations were developed from the procedure of Goede (1996), and parts of this guide are cross-referenced to his atlas of photographs (Goede 1988). The guide has been made more generally applicable (i.e., for use in a wider geographic area and in other programs and studies) by eliminating references to specific collection locations and cooperators; by providing for the collection of tissues and fluids not specifically required for the BEST pilot studies; by allowing for the collection and examination

of a wider variety of fishes; and by eliminating references to specific brands and trade names of supplies and equipment. Collection locations and times, preferred taxa, exact numbers of samples, modifications or additions to standard procedures, and field and laboratory cooperators should be identified in the study plan or protocol under which the procedures described in this guide are used.

Although the field procedures described in this guide can be used to obtain tissues and fluids with which to perform many assays, they were designed specifically for conducting the quantitative health assessment and for collecting the biological materials needed for the specific methods being evaluated in the 1995 and 1997 BEST pilot projects. Consequently, the methods described here have been used by the authors to conduct only these and similar assays, and the procedures may need to be altered by investigators using the guide to collect tissue and fluid types and quantities that meet the needs of other study plans or protocols. Corresponding changes may also need to be made in the lists of required equipment and supplies.

1.3 Typographic Conventions

Within this document, the following typographic conventions are used:

- Important actions, notes, and reminders are indicated in **bold** or *italics*.
- Information to be recorded is also indicated in **bold**.
- The cross symbol (✚) indicates a health- or safety-related item.

1.4 Animal Welfare

The procedures described in this guide conform to the American Society of Ichthyologists and Herpetologists (ASIH), American Fisheries Society (AFS), and American Institute of Fishery Research Biologists (AIFRB), "Guidelines for Use of Fishes in Field Research" (ASIH, AFS, and AIFRB 1988) and with all known SOPs and guidelines for the humane treatment and disposal of test organisms during culture and experimentation.

2.0 Roles and Responsibilities

Generally, studies based on this guide will have both field and laboratory components, of which the guide describes only the field portions. Conducting or otherwise arranging for necessary laboratory analyses is usually the responsibility of the principal investigator, project coordinator, or study director. Activities and responsibilities of field cooperators and personnel include: coordinating and conducting sampling operations; ensuring that personnel are trained in the operation of electrofishing, GPS, and photographic equipment (which are beyond the scope of this guide); conforming to all pertinent health, safety, certification (watercraft operation,

electrofishing, etc.), and collection permit requirements (both state and federal collection permits and their required reporting and pre-notification); securing prior written permission to enter private lands (see Section 2.1 below); ensuring that boats, motors, trailers, and other equipment are properly cleaned after sampling (to thwart the transport of living organisms between waterways); and shipping samples and records to cooperators and laboratories. Consult the study plan to determine the species and number of specimens required; this information is necessary under the terms of many collection permits.

Note: At least one representative of each field team should have documented firsthand experience in the conduct of the procedures described in this guide.

2.1 Landowner Permission.

If travel across or sampling on private property is necessary, obtain permission from the landowner or a representative of the landowner, and have them sign and date a sheet prepared for that station (referred to as a Station Identification Sheet; see Appendix A for an example) to indicate landowner consent in advance of each entry.

2.2 Shipment of Samples and Records.

Frozen samples must be shipped to cooperating laboratories by overnight express in dry ice or in a liquid nitrogen dry-shipper. Because of the limited holding time of dry ice, it is best to sample early in the week and to ship no later in the week than Thursday morning. Liquid nitrogen dry-shippers, if well-charged, can be shipped on Fridays. Restrictions on shipping histopathology samples will depend on the fixative used. Check with shipping companies - shipping by ground transport in properly labeled boxes will likely be required.

Project participants should consider using chain-of custody (COC) forms when shipping or mailing samples and data, even if it is not required. An example of a COC form is enclosed within this document (Appendix B). All COC forms must be signed and dated in ink. See Section 6.0 for further details.

2.3 Shipment of Equipment and Supplies.

When sampling multiple sites with multiple teams, the use of non-expendable equipment (i.e., dry ice and/or liquid nitrogen shippers, balances, centrifuges) should be coordinated among cooperators in order to decrease costs. To expedite the flow of non-expendable equipment, it is important to plan schedules as far in advance as possible. This will help to ensure that the equipment reaches each team when they need it!

2.4 Determining the Age of Specimens.

Information on the age of each fish is important for interpreting the results of certain biomarkers.

This document does not specify procedures for determining the age of specimens collected. Because the particulars of aging fish vary among taxa and locale, field biologists are encouraged to consult local experts who specialize in aging the species to be collected before sampling begins.

2.5 Photographs and Video Tape.

Photographs or color slides of sampling sites and operations are desirable, both for presentations and as a record. It is also advantageous to photographically record any lesions or other anomalies identified during the fish health assessment, as noted in the instructions.

Note: It is generally better (in terms of quality and expense) to take multiple photos than to duplicate slides. Video tape is an acceptable alternative to film.

2.6 Communication.

Field biologists should have access to project coordinators / study directors in the event they need assistance. Pertinent telephone (including cellular telephone) and FAX numbers and email addresses should be provided.

3.0 EQUIPMENT AND SUPPLIES

The following equipment and supplies are needed to conduct the procedures outlined in this guide (Table 1). The number needed per station or per fish is indicated in parentheses ( ) unless that number must be determined from the study plan. The size of sample containers and of syringes may also need to be determined from the study plan.

Table 1. Equipment and Supplies

Items Needed for Each Station (regardless of the number of fish to be collected):

110V AC power source (1 inverter, generator, or outlet)
Acetone (0.5 L, for rinsing instruments)
Acetone squirt bottle w/ extra 28-mm lid for shipping and transporting (1)
Alcohol wipes (50, for hand cleaning)
Aluminum foil (300 ft, heavy duty)
Aluminum storage canes for cryogenic vials (total number varies with study plan)
Ballpoint pens (3)
Beaker (1, 100-ml, for acetone rinse)
Bleach (optional, for disinfecting equipment)
Blunt instrument for subduing fish (1)
Calibration weights (1 of appropriate mass for each balance used)
Camera and film, or cam-corder and tape (1)
Cellular telephone (optional, but recommended)
Centrifuge (1, only if plasma is to be collected)
Centrifuge tube rack (1, plastic or styrofoam)
Chain-of-custody forms (total number varies with study plan)
Clipboard (1)
Composite Sample Sheet (1)
Coolers
    Small (1, for quick-freezing)
    Large (total number varies with study plan, for cooling fish, etc.)
Cryogenic gloves (1 pr)
Cryogenic markers (2, for marking cryogenic vials)
Cryogenic vial storage boxes (total number varies with study plan)
De-ionized water (4 L, for rinsing instruments)
Dissecting tools (1 kit)
Dry ice (4-5 lb for freezing & 10-15 lb for shipping)
Dry ice shipper (1)
Duct tape (1 roll, for securing sharps containers, etc.)
Electrofishing boat, backpack shocker, or electric seine and related equipment
Electronic balances (3, capacities vary with study plan)
Ethanol, 100% (1.5 L; 95% may be substituted if necessary)
Field guide(s) or other reference(s) for identifying fish
Field notebook (1)
First-aid kit (1)
Flashlights and other lights (optional)
Garden sprayer or pump spray bottle (optional, for dispensing bleach solution)
GPS unit (1)

Ground-Fault Interrupting (GFI) extension cord (1)
Guide (1 copy)
Hand-lens (1)
Hanging balance, for large fish (1)
Heparin solution (sodium salt, 10 USP units/ml; 2 × 5-ml. Use is optional depending on study plan) -- ***Refrigerate/chill until used!***
Ice (50 lb)
Kitchen shears or scissors (1 pr)
Latex gloves (40 pr)
Liquid nitrogen (about 15 L, for freezing/shipping samples)
Liquid nitrogen dry-shipper (1)
Mailing labels (1 sheet)
Material Safety Data Sheet for fixative (1)
Measuring board, mm (1)
Paper towels
Plastic bags (total number varies with study plan, enough for composite fish carcass samples)
Plastic trash bags (for waste disposal)
Pliers (1 pr)
Portable table (optional)
Rigid-walled container w/lid (i.e., coffee can, milk jug) for sharp wastes, or commercially procured sharps container (1)
Safety glasses (2-3 pr)
Shipping labels for samples (total number varies with study plan)
Station Identification Sheets (1)
Slotted serving spoon (1)
Small towels or cloth diapers (6)
Stainless steel pan for weighing fish < 2000g (optional)
Study plan or procedure (1)
Tubs or live boxes (if boat has no livewell)
Waterproof marking pens (3)
Waterproof paper for labels (2 sheets)
Work gloves (2 pr)

Items Needed for Each Fish (number may vary depending on study plan):

Bottles, 125-ml polyethylene (1 per fish, for histopathology samples)
Cloth tags (1 per fish and 2 per composite sample, for fish carcasses)
Cryogenic vials (preferably with color-coded caps or inserts)
    2.0-ml (2 per fish, for plasma or serum)
    1.2-ml (2 per fish, for liver tissue)
Fish Examination Sheets (1 per fish)
Fixative (85 ml per fish, for histopathology samples)
Needles (1 per fish; size may vary depending on fish species and size)

Scale envelopes (1 per fish, for scales or spines)
Syringes
    5.0-ml (1 per fish)
    3.0-ml (1 per fish)
Transfer pipettes (1 per fish, for plasma or serum)
Vacuum containers (1 per fish, for blood; heparinized or unheparinized, depending on study plan)
Weigh boats (1 of each per fish, small and large)

4.0 RECORD KEEPING, AND HOUSEKEEPING

Activities to ensure proper record keeping are outlined in Box 1. Housekeeping activities that should be performed throughout the sampling day are outlined in Box 2.

Box 1. Suggested Activities to Ensure Proper Record Keeping.

- Prepare and retain a short-form resume (see Appendix C for an example) for all personnel involved in sampling.

- Keep a logbook of the team's activities (when the team left, who was present and what each person did, etc.).

- **Make all entries in ink. Use a ballpoint pen, which will write under all conditions on data-entry and other forms. Forms should be printed on waterproof paper.**

- PRINT legibly.

- Do not use "ditto" marks (") for observations that repeat on a series of lines. Instead, write the entry on the first and last applicable lines, and connect the observations with a single vertical line.

- If you do not attempt a measurement (equipment broken, etc.), write an explanation in the comment field on the data sheet.

- **If you make a mistake, draw a <u>SINGLE</u> line through the incorrect entry, and initial and date the correction. Then, indicate the nature of the error in the appropriate comment field on the data sheet.**

- Number sampling sites in accordance with the protocol or study plan under which an investigation is conducted. Each sampling site should have a unique identifier. **Initiate a new Station Identification Sheet for each day that you sample at a site.**

- Number fish collected at a sampling site as instructed by the protocol or study plan. If no numbering system is detailed in the protocol, fish should be numbered with a unique identifier (referred to as the fish identification number) that indicates the ID of the individual specimen, the sampling site from which it was collected, and the date of collection.

- **Initiate a new Fish Examination Sheet (see Appendix A for an example) for each specimen.** Record the species, sex, length, weight, results of the field health assessment procedures, numbers of tissue and fluid samples collected, and any further remarks or comments pertaining to individual fish. To eliminate possible bias in subsequent analyses, samples bear the fish identification numbers, but no other markings.

- If you photograph (or videotape) any external or internal lesions or anomalies, use a previously prepared tag or scale envelope as a specimen identifier in each frame. There are spaces for recording the number of photos taken or the video footage shot in each section of the Fish Examination Sheet; filling them in will make it easier to annotate slides or tape later. It is easier for the recorder if you follow the order of the Fish Examination Sheet, which follows the order of this document.

- If carcass samples are composited for analysis, use the numbering system designated in the study plan or protocol. The system should be designed so that it does not conflict with fish identification or sampling site numbering schemes. Codes that include numbers and letters have been used successfully in the past. Group the samples as outlined in the study plan. The composition of composite samples should be recorded on a Composite Sample Worksheet (see Appendix A for an example).

  Note: If large specimens that are designated as juvenile, indeterminate, or undeterminable are collected and gender identification will be made histologically at a later date, group and bag these specimens separately.

- **Label cryogenic vials *only* with cryogenic markers, which are both water- and alcohol-resistant! Do *not* use a pen that is only waterproof!** Label all other containers (except the cryogenic vials) with a permanent, waterproof, felt-tipped pen. If in doubt, smell the marker; waterproof markers that are not alcohol-fast smell like solvent.

  Note: Cryogenic vials with white label fields are preferred over those without label fields. Write in the label field if there is one. Stick-on labels or label tape cannot be used if the dry-ice/ethanol freezing procedure described in this guide is used; the ethanol will dissolve the adhesive.

- Check the Fish Examination Sheets carefully to be certain that all observations have been made and recorded before proceeding to the next fish. Do not leave blank fields on the data sheets unless specified by the study plan.

Box 2. Suggested Housekeeping Activities, to be Performed as Needed.

- Place disabled needles and syringes in the sharps container; place used expendables in a trash bag.
- Cap the acetone squirt bottle and the marking pens when they are not in use.
- If collecting plasma, centrifuge blood samples; remove and freeze plasma.
- If collecting serum, allow blood to clot [on (wet) ice]; remove and freeze serum.
- Check dry ice and slush; refresh as necessary.
- Transfer frozen liver samples from the slush to the shipping container (either storage box(es) in the dry ice cooler or aluminum canes in the liquid nitrogen dry shipper).
- **After each fish is processed, thoroughly wash all contact instruments and dissecting tools and rinse them with acetone.**

5.0 FIELD PROCEDURES

The procedures described for processing fish in Section 5 are diagramed as flow charts that are referred to through Sections 5 and 6. Flow Chart 1 (Master) represents an overview of the complete fish-processing procedure.

5.1 Before Leaving on a Collecting Trip

a) If time permits, pre-label tags, labels, envelopes, and containers (Figure 1). Label a number appropriate for the number of specimens you will be collecting given the specifications of the study plan. **Bring extra, unlabeled supplies.**

Note: It is also advantageous to label, group, and package the expendables needed for each fish prior to arrival on site. Using the list of items needed for each fish (Table 1), put the following in a zippered plastic bag: needles and syringes (one each, 3.0- and 5.0-ml), one vacuum container, labeled, 1.2- and 2.0-ml cryogenic vials (two of each), one scale envelope, and one labeled cloth tag. These numbers may vary with the study plan; consult it before packaging.

b) Pre-fill the 125-ml bottles with about 85 ml of fixative.

- **10% neutral-buffered formalin is among the preferred fixatives because it can be shipped via ground transport. The fixative may vary with the project; therefore, consult the study plan. Regardless of the fixative used, it should be handled with respect. All personnel should read the Material Safety Data Sheet (MSDS) for the fixative being used and a copy of the MSDS should accompany the team in the field. Fill containers under a hood or outdoors, and wear protective clothing (lab coat or long sleeves and pants; latex or vinyl gloves; and safety glasses, goggles, or face shield) to prevent eye and skin contact.**

c) Check the equipment list to be certain all materials and equipment are present, and check the operation of all equipment to ensure that it is functioning properly (Table 1).

d) All equipment should be clean when it is taken out into the field -- clean any equipment that needs it. Any material that will come in contact with specimens should be cleaned with cleaning agents that will not affect later analyses.

e) Fill the dry ice shipper with dry ice.

Note: Blocks are better than cubes. Also, if you will be shipping samples in dry ice, bring extra in additional coolers.

f) If a liquid nitrogen dry shipper is to be used, get it filled.

✚ **Safety notes concerning cryogenic refrigerants (i.e., liquid nitrogen and dry ice):**

Liquid nitrogen and dry-ice are dangerously cold. Dry ice should be handled with tools (preferably), cryogenic gloves, or both. Personnel handling liquid nitrogen-filled dewars and dry shippers should wear long sleeves and pants, face shield or eye protection, and gloves. In addition to the obvious cold hazard, these cryogenic refrigerant gasses also represent an insidious hazard; because of their low temperature, they are denser than ambient air and, as they change from the solid (dry-ice) or liquid (liquid nitrogen) to the gas phase, the gasses tend to sink. Although neither carbon dioxide nor nitrogen are toxic, they can and will displace air containing oxygen. Moreover, containers for these refrigerants are designed to vent to the atmosphere. Consequently, these materials and containers should be stored in well-ventilated areas when possible. If liquid nitrogen dry shippers are stored in walk-in freezers when charged, personnel should be notified that the composition of the air in the freezer may be affected by the presence of the refrigerant gasses. A notice to this effect should be posted on the freezer door.

5.2 General Procedures to be Followed Upon Arrival at a Station

The preferred minimum size of a sampling team is six individuals. Once at the station, do the following before you start sampling:

Divide the team into two groups (of at least three), if possible. One group (Group 1 -- fish processors) should set up the sampling equipment, label containers (if not already done), and process fish while the second group (Group 2 -- fish collectors) collects and ferries fish to the processing station.

Group 1, which includes the trained diagnostician and a recorder (Figure 2), should:

a) Initiate the Station Identification Sheet for the day's sampling by **recording the station number, date, station description, start time, freezing method being used, and the names of the team members**.

b) On the first day of sampling at a station, initiate a Composite Sheet for composite samples by **recording the station number and date**.

Note: It is suggested that one person be designated as the recorder for the duration of the sampling at any site.

c) Find the most level, shady, wind-sheltered place possible to set up the balance and centrifuge, and provide 110-V A.C. power for this equipment (i.e., inverter and 12-V D.C. boat, vehicle, or trolling motor battery; portable generator; electrofishing boat; or a grounded 110-V A.C. outlet) (Figures 3a, 3b, 3c).

✚ **Important! A Ground-Fault Interrupting (GFI) device is required by the Occupational Safety and Health Administration (OSHA) when working from a portable, ungrounded generator. It is highly recommended when working with A.C. power outdoors at any time. Purchase an extension cord with a GFI, if you do not already have one, and use it at all times when working with A.C. in the field!**

A portable work surface (e.g. folding table, tailgate, cooler top) is also helpful (Figure 3c).

d) Set up and level the balances (Figure 3a).

Note: Setting the balances up inside an empty cooler on a level spot works well. If it is windy or raining, close the lid between operations.

e) Power up the balance(s) and ascertain that it (they) is (are) operating properly by weighing the appropriate calibration mass. **Record the make, model, and ID or serial number of the balance(s) and the "Before" calibration weight(s)** on the Station Identification Sheet. All weights should be recorded in grams and the recorder should confirm that the balance is in the desired mode at all times. Also, personnel working with the balances should ensure that any weigh boats or other equipment being used to hold fish or organs are resting completely and solely on the weighing plate of the balance. Check the zero of the hanging balance, and adjust if necessary using the thumb screw at the top of the cylinder.

f) Unpack the expendables (cryogenic vials, centrifuge tubes, bags, tags, labels, etc.) (Figure 4), and label the containers (if not done already).

g) Fill a container (tub, bucket, cooler) with ambient water and pre-soak the diapers or towels to be used to handle the fish (to remove any detergents or other chemicals).

h) Keep heparin, syringes, and vacuum containers chilled on ice until needed.

✚ **For protection against pathogens and parasites associated with either the fish or water, disposable latex or vinyl gloves should be worn by team members handling the fish and collecting tissues and fluids. The gloves should be replaced as necessary if they become torn or**

punctured, and disposed of properly. Hands should be washed with disposable alcohol wipes.

Group 2 (fish collectors) should:

a) Obtain the Station Identification Sheet and **record settings for the electrofishing equipment (in the General Comments) and location coordinates**. Then, initiate sampling (Figure 5). **Record the range of latitude and longitude sampled in degrees (000°), minutes (00'), and seconds (00") and the stop time** when finished.

5.3 Collecting and Holding Fish

The target taxa and desired number of specimens should be found in the study plan or protocol for the specific project. The collection of larger, adult fish is encouraged, for three main reasons: first, obtaining the necessary amounts of blood and tissues for the procedures outlined here may be difficult with smaller, juvenile fish; second, many biomarkers are neither valid nor well-documented in juvenile fish; and third, the observations called for may be difficult to perform with small fish. The latter is especially true for determining fish gender and weighing organs in the field. For studies that require identification of fish gender, specimens large enough to be mature but which are of indeterminate gender may be used if gender can and will be determined histologically (using preserved tissues) at a later time.

The preferred collection methods are D.C. electrofishing (Figure 5), seining, or D.C. electric seining (the methods of capture that are least injurious to the fish); however, be certain that nets have not been chemically treated or used previously in heavily contaminated waters. Hook-and-line capture is also permissible. To complete the procedures described here, **fish must be held alive.** Trap-, gill-, or trammel-netting, and A.C. electrofishing should not be used because these methods are more injurious to the fish and can bias the quantitative health assessments and biomarker analyses.

Note: Holding fish for prolonged periods can affect biomarkers and health assessments; you should begin processing specimens before finishing the collection. Although predatory fish may be more available for capture in the evening, holding fish overnight for next-day processing is not encouraged because it may bias physiological parameters and biomarkers to an unknown degree. In addition, holding fish for prolonged periods in tanks or live wells with abrasive surfaces or in net pens can cause external lesions.

Note: It is seldom possible to ascertain the sex of a fish by external observation. Therefore, if a given number of each sex is desired, more than the target number of fish will have to be collected to meet the objectives of the study.

5.4 Preparing Slush for Quick-Freezing

When the first fish is ready to be processed, prepare the dry ice slush as follows:

Place a brick-sized block of dry ice in a small cooler. Gradually add about 1/3 the cooler volume (ca. 400 ml) of 100% ethanol and stir (with the slotted spoon) to make slush (i.e., snow-cone consistency). If the dry ice available is too solid for slush, use chips of dry ice to super-cool the ethanol solution. Ethanol can be re-used from one station to the next.

- **As noted earlier, liquid nitrogen, dry ice, and the dry ice-ethanol slush are dangerously cold; avoid skin and eye contact. Personnel handling samples and containers with the cryogenic coolants should wear long pants and sleeves, eye protection, and cryogenic gloves.**

- **Avoid splashing or touching the slush!**

- **Ethanol is also flammable.**

Close the cooler lid (loosely) and place the small cooler in a large cooler filled with (wet) ice. Open only when necessary. Check the wet and dry ice regularly and replace as needed. You should be able to stand the cryogenic vials in the slush; if you can't, add more dry ice.

Note: Samples in cryogenic vials should be quick-frozen in either slush or dry ice. Liquid nitrogen may be used in lieu of theses procedures; however, the operation of liquid nitrogen dewars is not addressed in this guide.

Note: In the event that dry ice becomes critical, abandon the slush, especially if you are going to ship or transport samples in dry ice. If you suspect that you will have to use dry ice from the shipper to maintain the slush, insert all cryogenic vials directly into the dry ice. Record the change in procedure on the Station Identification Sheet under Freezing Method Used. It is best to know in advance where you can obtain more dry ice.

5.5 Blood Collection and Initial Fish Processing (Flow Charts 1 and 2)

Note: This guide only describes drawing blood from the posterior caudal artery and vein. Another blood sampling location and method (e.g. , cardiac puncture) may be specified in the study plan. Within a study, be consistent in the location and method of blood collection.

- **This is a reminder that personnel handling and dissecting the fish should wear gloves.**

a) Prepare the work surface by covering it with a piece of foil (dull side up/shiny side down) large enough to wrap the fish you will be processing.

b) Secure a towel or diaper (from the soaking container). Wring it dry, then tare it on the large balance.

c) Assemble the needle and syringe (if necessary). Generally, use 3.0-ml syringes for fish ≤ 300 mm TL, and 5.0-ml syringes for fish > 300 mm TL.

Note: If serum is to be obtained rather than plasma, no anticoagulant will be used. Skip to step (e).

d) Heparinize the needle and syringe by uncapping the needle and drawing a few milliliters of the heparin solution provided into the syringe, inverting it (i.e., needle up), and withdrawing the plunger to its full extent. Dispense the heparin back into its container. The small amount remaining on the walls of the syringe and in the hub of the needle will prevent clotting for most fishes. The exception is the black basses and other sunfishes (Centrarchidae), the blood of which clots readily. For these fishes, leave a visible meniscus (ca. 0.2 ml) in the syringe.

✚ **Heparin is a powerful anti-coagulant -- be careful! Also, carefully assemble and disassemble needles and syringes.**

e) Secure a fish from the holding tank. Again, ascertain its condition (i.e., that it is alive) and identify it to species. **Make a reasonably positive identification at this time**, using a guide to species names and abbreviations. A subset of species names and codes for fish that are most likely to be used with these procedures can be found in Appendix D. Initiate a Fish Examination Sheet for the fish by assigning the fish an ID number. **Record the Station ID number, date, fish ID number, and species** on the Fish Examination Sheet. If you are not sure of the fish species identification, note on the data sheet that you will confirm it at a later time.

Note: The authority for fish nomenclature is Robins et al. (1991).

f) To collect blood, wrap the fish securely in the towel or diaper, leaving the caudal peduncle exposed. The posterior caudal artery and vein lie together just ventral to the vertebral column. These vessels can be reached with a needle and syringe inserted between the hemal arches and spines from either of two directions:

> **Lateral approach (Figure 6):** Lay the fish on its (right) side, head elevated. Remove a few scales from the lateral line near the anal fin. Insert the needle from the side, just below the lateral line, to the approximate mid-sagittal depth.

Ventral approach (Figure 7): Hold the fish on its back, head elevated, with the ventral surface facing you. Remove a few scales from the narrowest part of the caudal peduncle, and insert the needle in the mid-sagittal plane. Push the needle toward the vertebrae (dorsally), angled anteriorly. *Note: This method does not work for all species.*

✚ **Be careful! Using a needle and syringe with an un-anesthetized, live fish can be dangerous. Don't stab yourself or a co-worker.**

Exert **slight** suction. When the needle contacts the base of the vertebrae (if drawing ventrally), withdraw the needle slightly. Too much suction will collapse vessels and tissues around the needle, restricting blood flow. It may be necessary to try more than once; if so, move anteriorly with each new attempt. However, repeatedly withdrawing the needle increases the likelihood of a clot forming. If a clot does form, withdraw the needle from the fish and expel the clot with slight pressure on the plunger of the syringe.

Fill the syringe with blood (if possible). **Record the volume of blood obtained**, as well as any problems encountered, on the Fish Examination Sheet.

Note: The minimum quantity of blood that yields a useable volume of plasma or serum should be specified in the study plan or protocol. Blood samples should be collected from a single location on all fish in a study to avoid introducing variation into the results.

g) Using your pliers, remove the needle from the syringe, crush or bend it, and put it in the sharps container for later disposal.

h) Remove the cap from a labeled, chilled, vacuum container. If the study protocol's procedures require serum, use a non-heparinized vacuum container. If the procedures require plasma, use a heparinized vacuum container. To avoid bubbles and hemolysis, **gently** dispense the blood from the needle-less syringe into the vacuum container--tilt the vacuum container slightly and run the blood down the side of the tube. Place the vacuum container in the tube rack cradled in ice (wet) and chill until processed.

i) Crush the syringe and place it in the trash bag for later disposal.

j) Conduct the external examination of the head surface and the eyes at this time (per instructions under Section 5.6.1 and 5.6.2). **DO NOT remove anomalies** at this time -- the fish has not been subdued.

k) Expose the anterior dorsal surface of the specimen and, with the blunt instrument of your choice, subdue the fish with a sharp blow to the rear of its head.

l) Place the fish, in its towel, on the previously tared electronic balance. If the fish is too

heavy for the balance, unwrap it and weigh it on the hanging balance. A tared holding container (e.g. a stainless steel pan) can be used to weigh fish < 2000 g on the hanging balance.

m) **Record the weight of the fish (± 1 g)** on the Fish Examination Sheet.

Note: Check the zero and the operational mode of the balances periodically between fish, and re-adjust or tare as necessary.

n) Unwrap the fish and measure it on the measuring board (total length, in millimeters; squeeze the caudal fins together - Figure 8).

o) **Record the total length (mm)** on the Fish Examination Sheet.

p) If you have not already done so, **label a tag and a scale envelope (fish identification number)** (Figure 1); use a permanent, waterproof felt-tipped pen and write in large, block letters. Use one or the other as a label in any photographs or video footage.

5.6 External Examination (Flow Chart 3)

Work quickly; the tissues need to be collected and frozen or preserved as rapidly as possible to avoid enzyme breakdown and tissue necrosis. Place the subdued fish on the measuring board to make general observations (Figure 9).

Record general remarks about fins, skin, and other external features on the Fish Examination Sheet before you begin the specific observations of particular organs, tissues, and structures. Important conditions to note are deformities, scale loss, external parasites, etc.

Note: Aesthetic observations are unnecessary.

Begin the external observations as outlined on the Fish Examination Sheet; use Appendix E as a guide. Within each section, be sure to **record all observations by marking (X) in all boxes on the data sheet that apply**. Recorded examinations of eyes, opercles, pseudobranchs, and fins may include marking both 'normal' and deteriorated conditions since more than one structure is involved.

Make a clear, precise mark for data entry for the external and internal examinations. If the observation does not seem to fit any of the listed categories, **check "Other" and describe your observations** in the indicated areas. **Record further remarks about listed categories** in the General Comments section.

Note: The general procedure during the external and internal examination is to record all observations that apply, and to collect and preserve a sample of anything that appears

abnormal. If an abnormal sample is collected, it is helpful to the histopathologist to also collect and preserve some normal tissue of the same type.

5.6.1 Body Surface. Examine all surfaces for any **Tumors, Lesions, or Parasites.** Remove them and place them in the labeled bottle of fixative.

Record anomaly type(s) found by checking the appropriate blocks. **Describe any anomalies noted as 'Other'. Note the general location of all anomaly type(s) in the "Other" field and record the number of pieces in fixative.**

5.6.2 Eyes (Goede 1988, Pictures 1 - 6). The left and right eyes should be examined and scored separately, as follows (check all that apply):

Normal -- No aberrations evident; eyes "clear," not protruding, milky, opaque, or bloody.

Exophthalmic -- Swollen, protruding eye. More commonly referred to as "pop-eye" or "goggle-eye."

Hemorrhagic -- Bleeding in the eye.

Opaque -- This is a very graphic category and you need not know whether the eye is functionally blind. It generally refers to opaque or cloudy eyes, or the appearance of cataracts. The nature of the opacity is not important here.

Missing -- An eye appears to be missing from the fish *(Note: it may actually be scarred over).*

Emboli -- Gas bubbles visible.

Other -- Any manifestations that do not "fit" the above (i.e., parasites, spots, cuts, abrasions); describe.

Record the condition of each eye and the number of pieces in fixative for each eye on the Fish Examination Sheet.

5.6.3 Opercles (Goede 1988, Picture 60). It is necessary only to observe the degree of shortening of the opercular flaps. Score the opercles according to the following criteria (check all that apply):

Normal -- No shortening; gills completely covered.

Slight shortening -- Slight shortening of the opercle with a very small portion of the gill exposed.

Severe shortening -- Severe shortening of the opercles with a considerable portion of the gill exposed.

Other -- Any observation that does not fit above; describe.

Record the condition of the opercles and the number of pieces in fixative.

5.6.4 **Gills** (Goede 1988, Pictures 7 - 15). The gills are examined and evaluated separately. Be very careful when observing the gills. Gill structure and tissue can easily be affected by the manner in which the fish is handled during and after collection. Evaluate the gills as follows (check all that apply):

Normal -- No apparent aberration.

Frayed -- This generally refers to actual erosion of tips of gill lamellae resulting in "ragged" appearing gills. Mere separation of gill lamellae can be construed as "frayed" but the condition may have been caused by something as simple as the manner in which the gill was exposed by the investigator.

Clubbed -- This refers to swelling of the tips of the gill lamellae. They can often appear bulbous or "club"-like.

Marginate -- A graphic description of a gill with a light discolored margin along the distal ends or tips of the lamellae or filaments. Margination can be and often is associated with "clubbing." If both seem to apply, check both.

Pale -- This refers to gills that are very light in color. Severe anemia can result in gills that are discolored to the point of being white. Severe bleeding induced during sampling of blood can also result in somewhat pale gills. Gills also begin to pale somewhat after death, which is common in fish taken from nets. All of this should be considered in making the observation.

Other -- Any observation that does not fit the above; describe.

Record the results of the examination of each gill on the Fish Examination Sheet.

Cut one small section of normal-appearing gill as well as any grossly visible anomalies and place them in the fixative.

Record the number of pieces in fixative for each gill on the Fish Examination Sheet.

5.6.5 *Note: Observation of the **Pseudobranchs** is to be performed **only for salmonid fishes**.* For all others,

Check the box marked "Other", write "NA" in the pseudobranchs comments, and proceed to the examination of the Fins (Section 5.6.6).

Pseudobranchs (Goede 1988, Pictures 16 - 23). Lift each opercular flap and locate the pseudobranchs, which lie dorsal and anterior to the gills. Score the pseudobranchs as follows (check all that apply):

Normal -- Concave or flat, with no visible aberrations.

Swollen -- Convex (not flat or concave).

Lithic -- Mineral deposits producing a granular appearance or white spots.

Hemorrhagic -- A general category that includes all forms of redness beyond the normal red color, which can be caused by hemorrhage, infection, etc.

Other -- Any observation that does not fit the above; describe.

Record the condition of the pseudobranchs and the number of pieces in fixative on the Fish Examination Sheet.

5.6.6 **Fins** (Goede 1988, Pictures 61 - 63). Eroded or "ragged" fins are a departure from normal condition and health. Previously eroded fins that are healed over and show no evidence of active erosion are considered normal in this assessment. The evaluation of fins is relative to the degree of active erosion in evidence. For the purposes of this procedure, the number and fin location involved is NOT significant; the fins are considered in aggregate when the animal's condition is scored by a histopathologist. If only one fin is displaying active erosion or other abnormalities, **record the observation and fin location**. If several fins are displaying erosion with unequal severity, **check all that apply and note in the fin comments** which fins are abnormal.

Note: Erosion usually involves both the fin membrane and rays, whereas fraying involves only the membrane.

Normal -- No active erosion, fraying, or hemorrhage. This includes previously eroded fins that are completely healed over.

Mild erosion -- Active erosion but no evidence of hemorrhage or secondary infection.

Severe erosion -- Active erosion with hemorrhage and/or evidence of secondary infection.

Frayed -- Margins of fins ragged or torn.

Hemorrhagic -- Reddened (i.e., bloody) areas visible within the intact fin.

Emboli -- Gas bubbles visible within the fin.

Other -- Any observation that does not fit above; describe.

Record the condition or conditions that apply if one or more fins is affected. **Note the location of any lesions** in the General Comments **and the number of pieces in fixative**.

5.7 Internal Examination and Sample Collection (Flow Chart 4)

Lay the subdued fish on the foil and open the abdominal cavity with a cut from the vent forward to the pectoral girdle; cut through or closely to one side of the pelvic girdle. Do **NOT** insert the scissors so far that the internal organs are damaged.

Note: You will be asked to weigh the liver (for some fishes), gonads, and spleen. Use the balance that will yield the requested number of significant digits based on the size of the fish and its internal organs.

Reach into the anterior end of the abdominal cavity with one gloved finger and find the esophagus. Cut the esophagus with the scissors, and remove the entire viscera except for the kidneys, which will remain in the carcass (Figure 10). Use only gloved fingers and a blunt probe to free the internal organs from the carcass. Put the carcass aside (on the foil) while working with the excised internal organs.

Begin the internal examination with the liver, which must be sampled and flash-frozen rapidly. Use Appendix E as a guide.

5.7.1 Liver, Gall Bladder, and Bile (Flow Chart 4a). Although treated separately here and on the Fish Examination Sheet, these three components are examined and sampled together.

5.7.1.1 Liver Weight

Note: The following apply only for fishes with a discrete liver (salmonids, largemouth bass, and others). For fish without a discrete liver (i.e. carp), write "NA" after Weight, and proceed to the Liver Observation (Section 5.7.1.2).

Tare a weigh boat or small piece of foil (dull side up/shiny side down) on the balance. Tease the liver and gall bladder free of mesenteric membranes with gloved fingers, and remove them together (Figure 11). Leave the intact gall bladder attached, and place the liver on the tared balance.

Note: Use the smallest capacity balance possible, based on the size of the fish and the liver.

Note: Should you inadvertently puncture the gallbladder, wash any contaminated liver, spleen, or kidney pieces with de-ionized water before putting them into the fixative or a cryogenic vial. If the contaminated parts of the organs are to be left in the fish or returned to the carcass later, they need not be washed.

Record the liver weight (± 0.1 g or less) on the Fish Examination Sheet.

5.7.1.2 Liver Observation (Goede 1988, Pictures 44 - 52). The appearance of the liver may be an artifact of the sampling; the observer should take that into consideration. Note that the appearance of the intact liver and gall bladder may, for example, vary with the length of time from collection to observation. It also depends on the extent of blood loss during sampling. Check all that apply.

Dark- to light-red color -- Dark red is the normal color. However, the liver is a blood storage organ, and it may be a lighter red color after bleeding, but not so pale as to be classified as general discoloration or as tan. Color may also vary among species and taxa.

Tan or "coffee with cream color" -- "Fatty" liver (i.e., more or less uniformly light tan color).

General discoloration -- Uniform color other than the above (gray is common); describe.

Focal Discoloration -- Color change in part of the liver, giving it a mottled appearance; describe.

Nodules in Liver -- Nodules (i.e., white parasitic cysts or bacterial granulomas) and incipient nodules, such as those in hepatoma (dark blotches) or cholangioma ("popcorn" look); swollen areas.

Other -- Aberration or deviation in the liver that does not fit into above scheme; describe.

Record the liver condition on the Fish Examination Sheet.

5.7.1.3 Liver Sample Collection. For fish **with** a discrete liver, use **acetone-rinsed scissors** to cut any grossly observable foci or lesions from the liver and put them into the fixative. **Try not to puncture the gall bladder**. Cut the remainder of the liver into 1-cm cubes in the weigh boat or on the foil.

Note: This line is 1-cm long. ____

Using **acetone-rinsed forceps**, fill labeled, 1.2-ml cryogenic vials (number specified in the study plan) about half full with liver tissue (Figure 12), and quick-freeze them in the dry-ice slush (you may use the slotted spoon). In addition to the grossly observable lesions (if any), place a minimum of five 1-cm cubes in the fixative for histopathological analysis. **Record the number of liver samples in fixative and the number of liver sample cryogenic vials** on the Fish Examination Sheet. Set the weigh boat or foil containing the remainder of the liver aside.

For fish **without** a discrete liver, such as common carp and goldfish, the procedure is analogous, but the liver cannot be removed and weighed. Upon completion of the visual observation, locate and inspect as many hepatic nodules as possible. *Note: Here, nodule refers to the dispersed liver organ, not to a pathological condition.* **Using acetone-rinsed scissors and forceps**, collect a minimum of five nodules that appear normal, cut them into 0.2-cm pieces, and try to fill the labeled, 1.2-ml cryogenic vials (number specified in the study plan) about half-full (Figure 12). Quick-freeze the cryogenic vials in the dry-ice slush. Collect any additional normal-appearing nodules, as well as all that appear abnormal, cut them into 1-cm pieces, and put them in the fixative. **Record the number of nodules (liver pieces) in fixative and the number of liver sample cryogenic vials** on the Fish Examination Sheet.

5.7.1.4 Gall Bladder Fullness and Bile Color (Goede 1988, Pictures 53 - 57). The bile is observed indirectly through observation of the gall bladder. The ranking scheme considers "fullness" of the bladder and degree of "green-ness", as follows:

> Bile color should be classified as
>
> **Yellow** or straw color;
> **Light green to grass green**; or
> **Dark green or dark blue-green**.
>
> Gall bladder fullness may be either
>
> **Empty** or nearly so;
> **Partly full**; or
> **Full or distended**.

Record the color of the bile and the fullness of the gall bladder on the Fish Examination Sheet.

Note: If bile color can be determined, bile is present and the gall bladder should be described as either "partly full" or "full".

5.7.2 Spleen (Flow Chart 4b) (Goede 1988, Pictures 31 - 33). Locate the spleen and dissect it whole from the viscera. Tare a weigh boat or small piece of foil. *Note: Use the smallest capacity balance possible based on the weight of the fish and the spleen.* Weigh the spleen and **record the spleen weight (± 0.002 g)** on the Fish Examination Sheet.

Observe the condition of the spleen as follows (check all that apply):

Red to Black -- "Black" is actually a very dark red color; both conditions are considered normal.

Granular -- Granular or "rough" appearance of the spleen.

Nodular -- The spleen contains or manifests fistulas or nodules of varying sizes. These are often cysts, such as those caused by parasites or chronic mycobacterial infections.

Enlarged -- The spleen can be significantly and noticeably enlarged.

Other -- Occasionally there are grossly visible aberrations that do not fit any of the classes above. The spleen may be mottled gray, and some fishes may have very small spleens. These should be classified as "Other" and described.

Record the spleen condition on the Fish Examination Sheet.

If the spleen is large (> 0.8 g), cut it in half and place it in fixative. Otherwise, place the entire spleen in fixative. **Record that the spleen in fixative was collected** on the Fish Examination Sheet.

5.7.3 Gonads (Flow Chart 4c). Locate the gonads and determine the gender of the fish, if possible. **Record the gender** on the Fish Examination Sheet (under the Gonads section and in the first section of page 1). Dissect the gonads from the viscera.

Tare a weigh boat or piece of foil on the appropriate balance. *Note: Use the smallest balance possible, based on the size of the fish and the gonads.*

Record the gonad weight (± 0.1 g or less) and the gonadal condition (i.e., ripe, spent, intermediate) on the Fish Examination Sheet. Ripe fish have distended abdomens and large, full gonads; ovaries contain many large eggs. The gonads of spent fish are small; ovaries contain at most a few small eggs, and the gonads are vestigial or nearly so. An intermediate condition is not distinctively either ripe or spent.

Note any lesions, parasites, etc. by checking "Other" (preserve a sample), and describe.

Cut 1-cm pieces from the posterior end (bottom tips) of the gonads, and place a

maximum of five gonad pieces in the fixative. If the gonad is small, preserve the entire organ.

Record the number of pieces in fixative, and set the weigh boat or foil containing the remainder of the gonad aside.

5.7.4 Mesenteric Fat (Goede 1988, Pictures 27 - 30). The ranking of mesenteric fat deposition was developed for salmonid fishes with prominent pyloric caeca. Pyloric caeca are also present in black basses (Centrarchidae). The following ranking system was developed for the rainbow trout but has been applied with minor variations to all major groups of salmonids, and has been adapted for the black basses here. For other fishes, check the mesenteric tissues in the visceral cavity for hemorrhage and inflammation. If these conditions are present, **record them** in the General Comments section on the Fish Examination Sheet.

Note: The following apply only for fishes with pyloric caeca (i.e., salmonids, centrarchids). For fishes other than salmonids and centrarchids, record hemorrhage or inflammation in the General Comments section (see above),write "NA" in the Mesenteric Fat section, and skip to the examination of the Kidney(s) (Section 5.7.5).

No Fat -- No fat deposited around the pyloric caeca and no fat deposited anywhere in the visceral cavity.

Slight Fat -- Slight is where less than 50% of each caecum is covered with fat.

50% -- about 50% of each caecum is covered with fat.

> 50% -- More than 50% of each caecum is covered with fat.

Completely covered -- Pyloric caeca are completely covered by a large amount of fat.

Record the mesenteric fat rating on the Fish Examination Sheet.

5.7.5 Kidney(s) (Flow Chart 4d). In some fishes (common carp, centrarchids), the anterior (head) and posterior (trunk) kidney are separate; in others (salmonids, ictalurids), they are continuous. We treat them as being together for examination, but separate for sample collection. Observe and sample both.

5.7.5.1 Posterior (Trunk) Kidney (Goede 1988, Pictures 36 - 43). The trunk kidney is exposed by removing the layer of mesentery at the posterior dorsal border of the abdominal cavity. Pull the swim bladder and some of the mesentery aside to expose the kidney. Observe its condition as follows (check all that apply):

Normal -- Firm, dark red color lying relatively flat dorsally in the visceral cavity along the length of the ventral surface of the vertebral column.

Swollen -- Wholly or partly enlarged or swollen.

Mottled -- Gray discoloration, mottled or "patchy" in appearance, ranging from scattered patches of gray to mostly or totally gray discoloration. This is not to be mistaken for the superficial gray appearance caused by the mesenteric membranes on the surface of the kidney. Move these membranes aside before examining and recording observations.

Granular -- The kidney has a "granular" or irregular surface which may be due to the presence of granulomas, hemorrhages, congestion or other causes. These areas are generally not hard and "gritty".

Urolithiasis -- Urolithic deposits are hard and "gritty". This condition is also known as nephrocalcinosis and involves deposition of white or "cream-colored" amorphous mineral material in the tubules of the kidney. It can range in appearance from very small white spots to severe conditions with very large "serpentine" deposits.

Note: These sites of deposition are not to be confused with the Stannius bodies or corpora of Stannius, which are the white nodular tissues present in salmonid kidneys and which have an endocrine function. The Stannius bodies are generally not associated with the tubules and usually occur at the "edges" in an area about midway along the kidney. They appear more globular than do urolithic deposits.

Other -- This is used to classify any aberrations that do not fit into the above scheme; describe.

Record the trunk kidney appearance on the Fish Examination Sheet.

Place at least one 1-cm piece of the trunk kidney in the fixative, and **record the number of pieces in fixative** (if possible--fish kidney tends to fall apart) on the Fish Examination Sheet.

5.7.5.2 Anterior (Head) Kidney. In fishes with separate kidneys, the head kidney is located dorsal to the liver and may be viewed after the latter has been removed.

Record the head kidney appearance according to the criteria above; record observations in the same section as above.

Remove as much of the head kidney as you can (at least one 1-cm piece) and place it in

fixative. **Record the number of pieces in fixative** (if possible) on the Fish Examination Sheet.

5.8 Scales or Spines (Flow Chart 1)

Collect a scale (most fishes) or pectoral fin spine (ictalurids) sample from the **left** side of the fish. If the left side is damaged (i.e., scales or pectoral fin not present), collect from the right side.

Spiny-rayed fishes (bass, walleye, etc.) -- from the area of the appressed pectoral fin (Figure 8a).

Soft-rayed fishes (common carp, suckers, etc.) -- beneath the anterior portion of the dorsal fin, above the lateral line (Figure 8b).

Ictalurids (i.e., catfishes) -- entire **disarticulated** pectoral spine; cut away as much fin and flesh as possible. Be sure to get the base of the spine (i.e., twist off like a chicken leg).

Note: These structures are included as representative because most fishery biologists have the training, experience, and equipment necessary to read them. Other hard structures (otoliths, opercular bones, vertebrae, etc.) may be used if these structures are known to be valid indicators of age in the species you are working with, and you have the capability to read them.

Place the scale or spine sample into a labeled scale envelope (Figure 1) and allow to air-dry. Store it until it can be read.

Record that the sample was collected and the side of the fish from which it was collected on the Fish Examination Sheet.

5.9. Preparing Carcass Samples (Flow Chart 5)

Prepare the carcass for chemical analysis by placing unused parts of the liver and gonads back into the abdominal cavity of the fish. To avoid fins poking through the aluminum foil, cut fin spines at base. Wrap the carcass securely in the aluminum foil that was used in the examination (dull side in/shiny side out). Large fish may need to be double-wrapped. Tie the previously prepared fish ID label (Figure 1) securely to the caudal peduncle (outside of the foil). Place the carcass sample in a large plastic bag of the proper composite (see study plan) and chill.

Initiate/Add the carcass sample to the tally of the appropriate composite sample (i.e., write the ID number of the individual fish) in the appropriate block on the Composite Sheet.

Note: Per earlier discussion, keep inter-sex or indeterminate individuals separate until

the gender has been confirmed histologically.

5.10 Processing Plasma or Serum Samples (Flow Chart 2)

5.10.1 Plasma. This procedure can be initiated at any time, but the centrifuge rotor must be balanced. Balance the rotor by spacing tubes evenly. If an even number of samples are not available, fill an empty tube with water and use it to balance the rotor. **Remove the caps** from the chilled vacuum containers to be centrifuged.

Centrifuge the tubes for 10 min @ 3500 rpm, then check the samples; the plasma should be transparent and straw-colored. If it is not, spin the samples for another 10 min. To avoid warming, don't go beyond 20 min total without re-chilling the sample(s).

Aspirate the plasma with a transfer pipette (Figure 3b); if necessary, **divide each sample** among the labeled 2-ml cryogenic vials (number specified in the study plan). Don't try to get every drop of plasma. It is better to leave a little in the cryogenic vials than to contaminate the plasma with debris. If you disturb the red blood cell pellet in the bottom of the tube, re-centrifuge the sample for a few minutes.

Securely cap the cryogenic vials, then **freeze them in dry ice (not slush due to potential ethanol contamination)**. Discard the excess plasma and pellet. **Record the number of plasma samples collected** on the Fish Examination Sheet.

5.10.2 Serum. Allow the blood samples to clot on (wet) ice. When the samples have completely clotted, **aspirate the serum** with a transfer pipette. If necessary, **divide each sample** among the labeled 2-ml cryogenic vials (number specified in the study plan). Do not disturb the clot. If you disturb the clot, allow the particulate materials to settle again before attempting to aspirate the serum.

Securely cap the cryogenic vials, then **freeze them in dry ice (not slush due to potential ethanol contamination)**. Discard the excess serum and clot. **Record the number of serum samples collected** on the Fish Examination Sheet.

5.11 After All Fish from a Station Have Been Collected and All Samples Have Been Processed

5.11.1 Packaging and Storing Composite Samples (Flow Chart 5)

Using a permanent, waterproof, felt-tipped pen, prepare a label [small (2-cm × 4-cm) piece of waterproof paper] and two tie-tags for each composite sample by writing the correct code on the label and both tags.

On the Composite Sheet, check the identity of the composite samples you have generated

at the station. **Double-bag** the **composite carcass samples**. Place the composite sample label in the outer bag, and **tie each bag** shut with a matching labeled tag. **The team leader** should **check to see that the tag codes match what is recorded on the Composite Sheet**. After confirming that the actual codes match those recorded on the sheet, initial the sheet in the "Reviewer's Initials" box. Store the composite samples in coolers with (wet) ice.

5.11.2 Complete and Check Fish Examination and Station Identification Data Sheets

Post-calibrate the balances by re-weighing the calibration masses, and **record the "After" calibration readings** on the Station Identification Sheet. Turn off the balance(s) and re-pack them in their case(s).

The **team leader** should **check the Fish Examination Sheets and the Station Identification Sheets again to be certain that all information has been recorded and is legible.** After each sheet has been reviewed and approved, it should be initialed in the "Reviewer's Initials" box.

5.11.3 Clean-up

Fasten the lid on the sharps container (tape it closed) and place it in the trash bag. Properly dispose of used expendables.

Thoroughly clean and inspect all equipment used in the field to protect against the transportation of living organisms among waterways. Procedures for cleaning equipment are beyond the scope of this document and may vary among agencies, regions, states, and water bodies. Pay particularly close attention to boats (including live wells), motors, trailers, and tubs used to hold fish. A stop at a car wash may be advantageous (be sure to rinse thoroughly with clean water). A garden sprayer or pump spray bottle containing a weak bleach solution can be used for a final rinse of all equipment.

Tap water is sufficient for cleaning smaller equipment (pans, measuring boards, coolers, live boxes), followed by the bleach solution. De-ionized water should be used to rinse dissecting equipment and the weighing pans of electronic balances (be careful!). Give dissecting equipment a final acetone rinse.

6.0 TRANSFERRING AND SHIPPING FROZEN LIVER, PLASMA, AND SERUM SAMPLES

If you are shipping liver samples in dry ice, use the slotted spoon to transfer any frozen liver samples in the slush bath to the plastic storage box(es), and transfer the boxes to the dry ice cooler. Transfer plasma or serum samples that have been frozen in dry ice to the plastic storage box(es) and back into the dry ice cooler. Keep the samples separated by type (liver and plasma

or serum).

If you are using a liquid nitrogen dry-shipper, transfer the frozen liver and plasma or serum samples to the dry shipper. Load the aluminum canes, and keep the samples separated by type (liver and plasma or serum). Load the cryogenic vials with the labeled surfaces facing outward so receiving personnel can read them.

When all samples have been transferred, empty the slush bath; as noted earlier, you may re-use the ethanol.

If liver, plasma, or serum samples will be shipped in dry ice, they should be shipped to cooperating laboratories or otherwise transferred to a -80°C freezer within 24 hours of collection. If you are visiting more than one station, this implies that you will have to ship samples before you return to your duty station. **Find out in advance how and from where you will be shipping samples, and plan your trip accordingly!** Samples in liquid nitrogen can be kept longer, depending on ambient temperatures and the length of time since the shipper was charged. Consult the study plan or protocol to determine the recipient(s) of frozen samples.

Note: It is essential that any receiving laboratory be notified by telephone in advance of shipping frozen samples. Speak directly to the designated individuals. Do not ship if you have only left a voice-mail message or sent a FAX or E-mail message.

Complete and sign a chain-of-custody form, and attach it and the addressed shipping label to the outside of the cooler (if shipping in dry ice). For liquid nitrogen dry-shippers, place the chain-of-custody inside the lid of the shipping case. Be sure that the dry shipper is properly labeled ("DRY SHIPPER, NON-HAZARDOUS") on the outside of the shipping container. **Before loading samples, be sure that the dry shipper contains no LIQUID nitrogen by tipping it on its side (outdoors). Shipping companies will not accept containers that contain any LIQUID nitrogen (see Appendix F).**

Note: Store the dry ice or liquid nitrogen shippers in a freezer if possible (remember that escaping refrigerant gasses may displace the air from the freezer!). Check dry ice regularly and supplement it as necessary. Most importantly, check your dry-ice or liquid nitrogen before you ship! If you are shipping samples on dry ice, remember to ship them by Thursday.

7.0 UPON RETURN FROM THE FIELD

7.1 Ship Frozen Liver, Plasma, and Serum Samples (per Section 7.0).

7.2 Process and Freeze Composite Fish Samples

Before freezing the composite fish samples, confirm the identity of any specimens you remain

unsure of; solicit expert advice if necessary. Make any necessary changes on the fish data sheets and re-bag samples as necessary to ensure the correct composition of each sample.

Be sure to **initial and date any corrections** you make on the Composite Sheet.

Freeze the composite samples, and store them frozen until they are ready to be shipped to the analytical laboratory. Ship composite samples in dry ice with a completed chain-of-custody form.

7.3. Photocopy and Mail Data Sheets and Other Records.

If the project / study director is stationed elsewhere, field teams should make two copies of all data sheets and field notes. One set of copies and the notebook should be retained by the team leader. The second set of copies and the original data sheets should be sent to the project coordinator / study director along with a completed chain-of-custody form. If the project coordinator(s) / study director(s) are members of the field team, they should retain both the original data sheets and field notes and at least one set of photocopies. For additional security, store the originals and copies in separate locations.

7.4. Ship Histopathology Samples.

Before you ship these samples, top off the fixative and check each bottle to be certain that the tops are tightly fastened. Samples should be shipped with a completed chain-of-custody form. **Check transport restrictions for your given fixative to determine the method of transportation.**

7.5 Process Film.

As soon as possible, **annotate your slides, photographs or videotape** - mark the station number, fish identification number (if applicable), and date.

7.6 Age Fish and Mail Age Data.

Fish should be aged using the techniques developed for the structure (e.g. scale, spine, opercle) that was collected. These techniques may vary with species. If the original data must be sent to a cooperating laboratory, original data sheets should be shipped with a completed chain-of-custody form. Chain-of-custody is optional if photocopied data sheets are being shipped.

7.7 Thoroughly Clean Non-Expendable Equipment and Supplies.

Equipment and supplies should be cleaned as soon as possible after returning from the field (see Section 5.11.3). Do not use detergents or other cleaning agents that could damage specimens or bias laboratory analyses during the next sampling trip.

Reminder: Special care should be taken when cleaning equipment to ensure that no living organisms are transported from one waterway to the next.

8.0 ACKNOWLEDGMENTS

In addition to the objectives stated in Section 1.1, the basin-wide sampling conducted in the 1995 and 1997 pilot studies also sought to evaluate the administrative and logistical feasibility of implementing the BEST Program through partnerships with other agencies and USGS programs. The studies were undertaken jointly by the BEST program, the Division of Environmental Contaminants of the FWS, and the USGS - NAWQA program. Field portions of the studies were coordinated and implemented by FWS Environmental Contaminants Specialists stationed at Ecological Services Field Offices and by NAWQA biologists, who were trained in the procedures described in this guide. We greatly appreciate the assistance of the FWS and USGS biologists and others who participated in the 1995 and 1997 pilot studies, especially those who provided input into the development of this guide; the BEST program for supporting the bulk of the 1995 and 1997 pilot studies and the preparation and publication of this guide; and the NAWQA program for supporting parts of the 1995 study. R.E. Jung and W.S. Bryant also contributed substantially to the development of this guide.

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11.0 APPENDICES

Appendices A-F follow.

APPENDIX E-16

STANDARD OPERATING PROCEDURE FOR U.S. EPA METHOD 8290 POLYCHLORINATED DIBENZODIOXINS (PCDDS) AND POLYCHLORINATED DIBENZOFURANS (PCDFS) BY HIGH-RESOLUTION GAS CHROMATOGRAPHY/HIGH-RESOLUTION MASS SPECTROMETRY [WSU-BURTON]

Wright State University
Brehm Research Laboratories
Standard Operating Procedure

for

U. S. EPA Method 8290

Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High-Resolution Gas Chromatography/ High-Resolution Mass Spectrometry (HRGC/HRMS)

Revision 2, July 1, 1999

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Scope and Application

U. S. EPA Method 8290 describes procedures for measuring the concentrations of tetra- through octa-chlorinated homologues of the polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in a variety of matrices at ppt to ppq concentrations. Brehm Research Laboratories (BRL) has implemented and validated modifications to Method 8290 that improve the accuracy and precision of the analytical measurements.

This SOP uses Method 8290 as the framework and incorporates the changes by referring to the appropriate sections. Method 8290 is included in Attachment 1.

The following items in Method 8290 are modified by this SOP.

- Lipid measurement procedure.
- Silica gel column.
- Alumina column.
- Charcoal column.
- Sample fortification, recovery, matrix spike fortification and calibration standards.
- Cleanup standard.

Definitions

Method 8290 – U. S. EPA Method 8290, SW-846, Revision 0, September 1994, "Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS)"

Method 1613 – U. S. EPA Method 1613, Revision B, 40 CFR Part 136, Appendix A, "Tetra-Through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS"

The definitions for this SOP are included in Method 8290 and Method 1613.

Procedures

Section 6.7.1, Lipid Content Determination – Fish Tissue

This section is modified to permit the use of a smaller quantity of the Soxhlet extract for the determination of the lipid content when less than 20 g of fish tissue is available for analysis. When 12 g to 20 g of tissue is available for extraction, the aliquot of extract that is removed for the lipid measurement is adjusted to leave the equivalent of a 10 g sample for GC-MS analysis. When less than 12 g of tissue is available for extraction, the lipid measurement is performed using 5% of the sample extract. The formula for the percent lipid calculation is the same as given in Section 6.7.1.

The portion of the tissue extract that will be used for the lipid measurement is determined before extraction so that the quantity of internal standard solution added in Section 7.1.2 can be adjusted to account for the removal of the lipid aliquot.

Sections 5.2 and 7.5.2, Silica Gel Column Cleanup

Silica gel, Type 60Å, 100-200 mesh (Davisil® 634, Mallinckrodt Baker, Inc., Paris, KY). The silica gel is extracted in a Soxhlet with methylene chloride for 24 hours and activated at 190 °C for 24 hours if blanks show contamination. If no contamination is observed, the silica gel is used without further activation. The following three types of silica gel chromatographic packing materials are prepared.

- Silica gel impregnated with 10% water is prepared by adding 10 g of HPLC grade water to 90 g of silica gel in a 500 mL wide mouth glass bottle fitted with a Teflon lined screw cap.
- Silica gel impregnated with sodium hydroxide is prepared by adding 30 g of 1 N sodium hydroxide solution to 100 g of silica gel in a 500 mL wide mouth glass bottle fitted with a Teflon lined screw cap.
- Silica gel impregnated with sulfuric acid is prepared by adding 44 g of concentrated sulfuric acid to 100 g of silica gel in a 500 mL wide mouth glass bottle fitted with a Teflon lined screw cap.

For each type of silica prepared, the bottle is shaken until no lumps are visible. After shaking, the silicas are transferred to 250 mL narrow mouth glass bottles fitted with Teflon lined screw caps. The silicas are transferred from the 250 mL bottles to the chromatographic columns immediately prior to use.

The silica gel column cleanup is performed on the concentrated extract from the acid/base partitioning procedure. The following procedure is used for the silica gel column cleanup.

1. Insert a plug of silanized glass wool into a clean glass column (20 mm OD x 230 mm long and tapered to a 6 mm tip).
2. Sequentially add the following to the column, tapping the column to settle and level the silica after each addition:

   1.0 g of water impregnated silica gel

   2.0 g of sodium hydroxide impregnated silica gel

   1.0 g of water impregnated silica gel

   4.0 g of sulfuric acid impregnated silica gel

   2.0 g of water impregnated silica gel
3. Preelute the column with 30 mL of hexane collecting the eluate in a waste collection tube.
4. When the hexane level reaches the level of the silica, change the collection tube to a cleaned 50 mL test tube.
5. Transfer the 5 mL of concentrated sample extract to the column.
6. When the level in the column reaches the level of the silica, sequentially rinse the sample container with two 5 mL portions of hexane and add each rinse to the column.
7. Elute the column with an additional 75 mL of hexane, collecting the entire eluate in two 50 mL test tubes.

8. Concentrate the hexane eluate to approximately 1 mL in preparation for the alumina column cleanup.

If the sample extract is still discolored after the four acid washes specified in Section 7.5.1.1 have been completed, then 10.0 g of sulfuric acid impregnated silica gel is used instead of the normal 4.0 g quantity in the silica gel column.

Sections 5.2 and 7.5.2, Alumina Column Cleanup

Alumina, basic, 50-200 µm, (ICN Alumina B, Activity I, ICN Biomedicals GmbH). The alumina is activated at 600±25 °C for more than 16 hours and less than five days in shallow ceramic pans.

The alumina column cleanup is performed on the concentrated eluate from the silica gel cleanup procedure. The following procedure is used for the alumina column cleanup.

1. Construct a disposable column by removing the portion of a 10 mL Pyrex disposable pipette above the 4 mL mark.
2. Insert a plug of silanized glass wool and carefully push it into the constricted end of the column using a 1 mL pipet.
3. Remove the alumina from the furnace, pour the alumina into a 150 mL Pyrex beaker and allow the alumina to cool in a desiccator for 30 minutes.
4. Mix the alumina in the beaker.
5. Add 3.0 g of alumina to each column and tap the columns to settle the alumina.
6. Transfer the concentrated sample extract from the silica gel column cleanup to the alumina column.
7. Sequentially rinse the test tube that contained the sample with two 1 mL portions of hexane and transfer each rinse to the alumina column.
8. Elute the alumina column with a total of 15 mL of hexane taking care to always maintain the level of the liquid in the column above the level of the alumina, and collect the eluate in a 25 mL test tube.
9. Elute the column with a total of 10 mL of 8% (v/v) methylene chloride-in-hexane, and collect the eluate in the test tube containing the hexane eluate.
10. Position a clean 25 mL test tube below the alumina column and elute the column with a total of 20 mL of 50% (v/v) methylene chloride-in-hexane.
11. Reserve the hexane/8% eluate and concentrate the 50% eluate to approximately 1 mL in preparation for the carbon column cleanup.

Sections 5.2 and 7.5.3, Carbon Column Cleanup

Active carbon PX-21 (Anderson Development Co., Adrian, MI) is prewashed with methanol, dried in a vacuum at 110 °C and stored in a glass bottle sealed with a Teflon lined screw cap.

The carbon column cleanup is performed on the concentrated 50% eluate from the alumina column cleanup procedure. The following procedure is used for the carbon column cleanup.

1. Prepare the packing material by thoroughly mixing 1.28 g of PX-21 carbon and 14.72 g of Celite 545 in a 250 mL glass bottle fitted with a Teflon-lined cap.

2. Prepare a disposable column by removing both ends of a 5 mL Pyrex disposable pipet at the 1 mL and –1 mL marks.
3. Cut a small disk from a GF/C glass fiber filter using a cork borer so that the disk fits tightly in the column and position the disk in the column at the 2 mL mark.
4. Add 0.30 g of the PX-21 carbon/Celite 545 mixture to the column and tap the column to settle the carbon.
5. Insert a second glass fiber filter disk into the column on top of the packing.
6. Position the column over a waste tube so that the end of the column containing the first filter disk is at the top.
7. Preelute the column in sequence with the following solutions and discard the eluates:

   4 mL of toluene

   2 mL of 50% benzene-in-ethyl acetate

   1 mL of 50% methylene chloride-in-cyclohexane

   2 mL of hexane
8. Transfer the concentrated sample eluate from the alumina column cleanup to the carbon column.
9. Rinse the test tube that contained the sample with 1 mL of hexane and transfer the rinse to the carbon column.
10. Elute the column sequentially with:

    2 mL of 50% methylene chloride-in-cyclohexane

    2 mL of 50% benzene-in-ethyl acetate
11. Reserve the eluate in the test tube and position a clean test tube below the column.
12. Invert the carbon column.
13. Elute the column with 6 mL of toluene, collecting the eluate in the clean test tube.
14. Using the toluene eluate, proceed with Section 7.5.3.6.

Section 5.8 and Table 2, Sample Fortification Solution

This nonane stock solution contains the fifteen internal standards at the concentrations listed below in SOP Table 1. This table partially replaces Table 2 in Method 8290. The solution contains a labeled internal standard for each 2,3,7,8-substituted PCDD/F except 1,2,3,7,8,9-HxCDD and OCDF. The identification and quantification procedures in Section 7 of Method 8290 are modified to use the additional internal standards. This stock solution is diluted prior to use.

SOP Table 1. PCDD/Fs in the Sample Fortification Stock Solution

PCDD/Fs	Concentrations in ng/mL
$^{13}C_{12}$-2,3,7,8-TCDD	100
$^{13}C_{12}$-2,3,7,8-TCDF	100
$^{13}C_{12}$-1,2,3,7,8-PeCDD	100
$^{13}C_{12}$-1,2,3,7,8-PeCDF	100
$^{13}C_{12}$-2,3,4,7,8-PeCDF	100
$^{13}C_{12}$-1,2,3,4,7,8-HxCDD	100
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	100
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	100
$^{13}C_{12}$-1,2,3,6,7,8-HxCDF	100
$^{13}C_{12}$-1,2,3,7,8,9-HxCDF	100
$^{13}C_{12}$-2,3,4,6,7,8-HxCDF	100
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	100
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	100
$^{13}C_{12}$-1,2,3,4,7,8,9-HpCDF	100
$^{13}C_{12}$-OCDD	200

Section 5.9 and Table 2, Recovery Standard Solution

This nonane stock solution contains the two recovery standards at the concentrations listed below in SOP Table 2. This table partially replaces Table 2 in Method 8290. These are the same labeled PCDDs that are used in Method 8290 but at different concentrations. The calculations using the recovery standards in Section 7 are not modified. This stock solution is diluted prior to use.

SOP Table 2. PCDDs in the Recovery Standard Stock Solution

PCDDs	Concentrations in ng/mL
$^{13}C_{12}$-1,2,3,4-TCDD	200
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD	200

Section 5, Cleanup Standard Solution

This nonane stock solution contains $^{37}Cl_4$-2,3,7,8-TCDD at 40 ng/mL. This standard is added to all sample extracts prior to cleanup to measure the efficiency of the cleanup procedures. The calculations for this cleanup standard are performed as specified in Section 17.2 of Method 1613. This stock solution is diluted prior to use.

Section 5.10, Matrix Spike Fortification Solution

This nonane stock solution contains the seventeen native 2,3,7,8-substituted standards at the concentrations listed below in SOP Table 3. These are the same PCDD/Fs that are required by Method 8290 but at different concentrations. This stock solution is diluted prior to use.

SOP Table 3. Native PCDD/Fs in the Matrix Spike Fortification Stock Solution

PCDD/Fs	Concentrations in ng/mL
2,3,7,8-TCDD	40
2,3,7,8-TCDF	40
1,2,3,7,8-PeCDD	200
1,2,3,7,8-PeCDF	200
2,3,4,7,8-PeCDF	200
1,2,3,4,7,8-HxCDD	200
1,2,3,6,7,8-HxCDD	200
1,2,3,7,8,9-HxCDD	200
1,2,3,4,7,8-HxCDF	200
1,2,3,6,7,8-HxCDF	200
1,2,3,7,8,9-HxCDF	200
2,3,4,6,7,8-HxCDF	200
1,2,3,4,6,7,8-HpCDD	200
1,2,3,4,6,7,8-HpCDF	200
1,2,3,4,7,8,9-HpCDF	200
OCDD	400
OCDF	400

Section 7 and Table 1, Addition of Standard Solutions to Samples and Extracts

SOP Table 4 lists the four standard solutions and the typical quantities of the standard stock solutions that are added to the various types of sample matrices.

SOP Table 4. Typical Quantities of Standard Solutions Added to Sample Matrices

Standard Solutions	Standard Added Prior to:	Typical Volumes of Stock Solutions Added to Sample Matrices [a]		
		Water	Soil, Sediment, Paper Pulp, Sludge, Fly Ash	Biological Tissue and Human Adipose
Sample Fortification Solution	Extraction	5 µL	10 µL	2.5 µL
Matrix Spike Fortification Solution	Extraction	25 µL	50 µL	25 µL
Cleanup Standard Solution	Cleanup	5 µL	10 µL	2.5 µL
Recovery Standard Solution	GCMS Analysis	2.5 µL	5 µL	1.25 µL

a. The volumes listed in this table are the volumes of the stock solutions that are added to the samples and extracts. The stock standard solutions are diluted, as required, before they are used.

Section 5.6 and Table 5, High-Resolution Concentration Calibration Solutions

Five nonane solutions containing native and labeled PCDD/Fs at known concentrations are used to calibrate the GC-MS. The concentrations of the PCDD/Fs in the five solutions are listed below in SOP Table 5.

SOP Table 5. Contents of the Five High-Resolution Concentration Calibration Solutions

PCDD/F	CS1 (ng/mL)	CS2 (ng/mL)	CS3 (ng/mL)	CS4 (ng/mL)	CS5 (ng/mL)
2,3,7,8-TCDD	0.5	2	10	40	200
2,3,7,8-TCDF	0.5	2	10	40	200
1,2,3,7,8-PeCDD	2.5	10	50	200	1000
1,2,3,7,8-PeCDF	2.5	10	50	200	1000
2,3,4,7,8-PeCDF	2.5	10	50	200	1000
1,2,3,4,7,8-HxCDD	2.5	10	50	200	1000
1,2,3,6,7,8-HxCDD	2.5	10	50	200	1000
1,2,3,7,8,9-HxCDD	2.5	10	50	200	1000
1,2,3,4,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,6,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,7,8,9-HxCDF	2.5	10	50	200	1000
2,3,4,6,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,4,6,7,8-HpCDD	2.5	10	50	200	1000
1,2,3,4,6,7,8-HpCDF	2.5	10	50	200	1000
1,2,3,4,7,8,9-HpCDF	2.5	10	50	200	1000
OCDD	5	20	100	400	2000
OCDF	5	20	100	400	2000
$^{13}C_{12}$-2,3,7,8-TCDD	100	100	100	100	100
$^{13}C_{12}$-2,3,7,8-TCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,7,8-PeCDD	100	100	100	100	100
$^{13}C_{12}$-1,2,3,7,8-PeCDF	100	100	100	100	100
$^{13}C_{12}$-2,3,4,7,8-PeCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,4,7,8-HxCDD	100	100	100	100	100
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	100	100	100	100	100
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,6,7,8-HxCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,7,8,9-HxCDF	100	100	100	100	100
$^{13}C_{12}$-2,3,4,6,7,8-HxCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	100	100	100	100	100
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	100	100	100	100	100
$^{13}C_{12}$-1,2,3,4,7,8,9-HpCDF	100	100	100	100	100
$^{13}C_{12}$-OCDD	200	200	200	200	200
$^{37}Cl_4$-2,3,7,8-TCDD	0.5	2	10	40	200
$^{13}C_{12}$-1,2,3,4-TCDD	100	100	100	100	100
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD	100	100	100	100	100

QC Limits

The QC limits are specified in Sections 7 and 8 of Method 8290.

Corrective Action Procedures

Method 8290 contains procedures for corrective actions.

Unforeseen situations may arise during the preparation and analysis of environmental samples. If possible, corrective action is implemented immediately and the actions are clearly documented on the sample tracking form or batch cover sheet.[3]

The need for corrective action may be identified by:

1. Recognition by laboratory personnel that there is a problem.
2. Standard QC procedures.
3. System or performance audits.

Usually, personal experience alerts the analyst to suspicious data or malfunctioning equipment. Corrective action taken at this point avoids the collection of poor quality data.

Problems that are not immediately detected during the course of analysis may require more formalized corrective action. The essential steps in the corrective action system followed by BRL are:

1. Checking the predetermined limits for data acceptability beyond which corrective action is required.
2. Identifying and defining the problem.
3. Assigning responsibility for investigating the problem.
4. Determining the action required to correct the problem (this may include reanalysis of the sample).
5. Assigning and accepting responsibility for implementing the corrective action.
6. Implementing the corrective action.
7. Verifying that the corrective action has eliminated the problem.
8. Documenting the corrective action taken.

References

1. U. S. EPA Method 1613, Revision B, 40 CFR Part 136, Appendix A, "Tetra- Through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS."
2. U. S. EPA Method 8290, SW-846, Revision 0, September 1994, "Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS)."

3. Quality Assurance Manual, Wright State University, Revision No. 7, March 23, 1998.

Attachment 1

U. S. EPA Method 8290, SW-846, Revision 0, September 1994

"Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS)"

POLYCHLORINATED DIBENZODIOXINS (PCDDs) AND POLYCHLORINATED DIBENZOFURANS (PCDFs)BY HIGH-RESOLUTION GAS CHROMATOGRAPHY/HIGH-RESOLUTION MASS SPECTROMETRY (HRGC/HRMS)

1.0 SCOPE AND APPLICATION

1.1 This method provides procedures for the detection and quantitative measurement of polychlorinated dibenzo-p-dioxins (tetra- through octachlorinated homologues; PCDDs), and polychlorinated dibenzofurans (tetra- through octachlorinated homologues; PCDFs) in a variety of environmental matrices and at part-per-trillion (ppt) to part-per-quadrillion (ppq) concentrations. The following compounds can be determined by this method:

Compound Name	CAS No[a]
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	1746-01-6
1,2,3,7,8-Pentachlorodibenzo-p-dioxin (PeCDD)	40321-76-4
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin (HxCDD)	57653-85-7
1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin (HxCDD)	39227-28-6
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin (HxCDD)	19408-74-3
1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin (HpCDD)	35822-39-4
1,2,3,4,6,7,8,9-Octachlorodibenzo-p-dioxin (OCDD)	3268-87-9
2,3,7,8-Tetrachlorodibenzofuran (TCDF)	51207-31-9
1,2,3,7,8-Pentachlorodibenzofuran (PeCDF)	57117-41-6
2,3,4,7,8-Pentachlorodibenzofuran (PeCDF)	57117-31-4
1,2,3,6,7,8-Hexachlorodibenzofuran (HxCDF)	57117-44-9
1,2,3,7,8,9-Hexachlorodibenzofuran (HxCDF)	72918-21-9
1,2,3,4,7,8-Hexachlorodibenzofuran (HxCDF)	70648-26-9
2,3,4,6,7,8-Hexachlorodibenzofuran (HxCDF)	60851-34-5
1,2,3,4,6,7,8-Heptachlorodibenzofuran (HpCDF)	67562-39-4
1,2,3,4,7,8,9-Heptachlorodibenzofuran (HpCDF)	55673-89-7
1,2,3,4,6,7,8,9-Octachlorodibenzofuran (OCDF)	39001-02-0

[a] Chemical Abstract Service Registry Number

1.2 The analytical method calls for the use of high-resolution gas chromatography and high-resolution mass spectrometry (HRGC/HRMS) on purified sample extracts. Table 1 lists the various sample types covered by this analytical protocol, the 2,3,7,8-TCDD-based method calibration limits (MCLs), and other pertinent information. Samples containing concentrations of specific congeneric analytes (PCDDs and PCDFs) considered within the scope of this method that are greater than ten times the upper MCLs must be analyzed by a protocol designed for such concentration levels, e.g., Method 8280. An optional method for reporting the analytical results using a 2,3,7,8-TCDD toxicity equivalency factor (TEF) is described.

1.3 The sensitivity of this method is dependent upon the level of interferences within a given matrix. The calibration range of the method for a 1 L water sample is 10 to 2000 ppq for TCDD/TCDF and PeCDD/PeCDF, and 1.0 to 200 ppt for a 10 g soil, sediment, fly ash, or tissue sample for the same analytes (Table 1). Analysis of a one-tenth aliquot of the sample permits measurement of concentrations up to 10 times the upper MCL. The actual limits of detection and quantitation will differ from the lower MCL, depending on the complexity of the matrix.

1.4 This method is designed for use by analysts who are experienced with residue analysis and skilled in HRGC/HRMS.

1.5 Because of the extreme toxicity of many of these compounds, the analyst must take the necessary precautions to prevent exposure to materials known or believed to contain PCDDs or PCDFs. It is the responsibility of the laboratory personnel to ensure that safe handling procedures are employed. Sec. 11 of this method discusses safety procedures.

2.0 SUMMARY OF METHOD

2.1 This procedure uses matrix specific extraction, analyte specific cleanup, and HRGC/HRMS analysis techniques.

2.2 If interferences are encountered, the method provides selected cleanup procedures to aid the analyst in their elimination. A simplified analysis flow chart is presented at the end of this method.

2.3 A specified amount (see Table 1) of soil, sediment, fly ash, water, sludge (including paper pulp), still bottom, fuel oil, chemical reactor residue, fish tissue, or human adipose tissue is spiked with a solution containing specified amounts of each of the nine isotopically ($^{13}C_{12}$) labeled PCDDs/PCDFs listed in Column 1 of Table 2. The sample is then extracted according to a matrix specific extraction procedure. Aqueous samples that are judged to contain 1 percent or more solids, and solid samples that show an aqueous phase, are filtered, the solid phase (including the filter) and the aqueous phase extracted separately, and the extracts combined before extract cleanup. The extraction procedures are:

a) Toluene: Soxhlet extraction for soil, sediment, fly ash, and paper pulp samples;

b) Methylene chloride: liquid-liquid extraction for water samples;

c) Toluene: Dean-Stark extraction for fuel oil, and aqueous sludge samples;

d) Toluene extraction for still bottom samples;

e) Hexane/methylene chloride: Soxhlet extraction or methylene chloride: Soxhlet extraction for fish tissue samples; and

f) Methylene chloride extraction for human adipose tissue samples.

g) As an option, all solid samples (wet or dry) may be extracted with toluene using a Soxhlet/Dean Stark extraction system.

The decision for the selection of an extraction procedure for chemical reactor residue samples is based on the appearance (consistency, viscosity) of the samples. Generally, they can be handled according to the procedure used for still bottom (or chemical sludge) samples.

2.4 The extracts are submitted to an acid-base washing treatment and dried. Following a solvent exchange step, the extracts are cleaned up by column chromatography on alumina, silica gel, and activated carbon.

2.4.1 The extracts from adipose tissue samples are treated with silica gel impregnated with sulfuric acid before chromatography on acidic silica gel, neutral alumina, and activated carbon.

2.4.2 Fish tissue and paper pulp extracts are subjected to an acid wash treatment only, prior to chromatography on alumina and activated carbon.

2.5 The preparation of the final extract for HRGC/HRMS analysis is accomplished by adding 10 to 50 µL (depending on the matrix) of a nonane solution containing 50 pg/µL of the recovery standards $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD (Table 2). The former is used to determine the percent recoveries of tetra- and pentachlorinated PCDD/PCDF congeners, while the latter is used to determine the percent recoveries of the hexa-, hepta- and octachlorinated PCDD/PCDF congeners.

2.6 Two µL of the concentrated extract are injected into an HRGC/HRMS system capable of performing selected ion monitoring at resolving powers of at least 10,000 (10 percent valley definition).

2.7 The identification of OCDD and nine of the fifteen 2,3,7,8-substituted congeners (Table 3), for which a ^{13}C-labeled standard is available in the sample fortification and recovery standard solutions (Table 2), is based on their elution at their exact retention time (within 0.005 retention time units measured in the routine calibration) and the simultaneous detection of the two most abundant ions in the molecular ion region. The remaining six 2,3,7,8-substituted congeners (i.e., 2,3,4,7,8-PeCDF; 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDF; 1,2,3,7,8,9-HxCDF; 2,3,4,6,7,8-HxCDF, and 1,2,3,4,7,8,9-HpCDF), for which no carbon-labeled internal standards are available in the sample fortification solution, and all other PCDD/PCDF congeners are identified when their relative retention times fall within their respective PCDD/PCDF retention time windows, as established from the routine calibration data, and the simultaneous detection of the two most abundant ions in the molecular ion region. The identification of OCDF is based on its retention time relative to $^{13}C_{12}$-OCDD and the simultaneous detection of the two most abundant ions in the molecular ion region. Identification also is based on a comparison of the ratios of the integrated ion abundance of the molecular ion species to their theoretical abundance ratios.

2.8 Quantitation of the individual congeners, total PCDDs and total PCDFs is achieved in conjunction with the establishment of a multipoint (five points)

calibration curve for each homologue, during which each calibration solution is analyzed once.

3.0 INTERFERENCES

3.1 Solvents, reagents, glassware and other sample processing hardware may yield discrete artifacts or elevated baselines that may cause misinterpretation of the chromatographic data (see references 1 and 2.) All of these materials must be demonstrated to be free from interferants under the conditions of analysis by performing laboratory method blanks. Analysts should avoid using PVC gloves.

3.2 The use of high purity reagents and solvents helps minimize interference problems. Purification of solvents by distillation in all-glass systems may be necessary.

3.3 Interferants coextracted from the sample will vary considerably from matrix to matrix. PCDDs and PCDFs are often associated with other interfering chlorinated substances such as polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers (PCDPEs), polychlorinated naphthalenes, and polychlorinated alkyldibenzofurans, that may be found at concentrations several orders of magnitude higher than the analytes of interest. Retention times of target analytes must be verified using reference standards. These values must correspond to the retention time windows established in Sec. 8.1.1.3. While cleanup techniques are provided as part of this method, unique samples may require additional cleanup steps to achieve lower detection limits.

3.4 A high-resolution capillary column (60 m DB-5, J&W Scientific, or equivalent) is used in this method. However, no single column is known to resolve all isomers. The 60 m DB-5 GC column is capable of 2,3,7,8-TCDD isomer specificity (Sec. 8.1.1). In order to determine the concentration of the 2,3,7,8-TCDF (if detected on the DB-5 column), the sample extract must be reanalyzed on a column capable of 2,3,7,8-TCDF isomer specificity (e.g., DB-225, SP-2330, SP-2331, or equivalent).

4.0 APPARATUS AND MATERIALS

4.1 High-Resolution Gas Chromatograph/High-Resolution Mass Spectrometer/Data System (HRGC/HRMS/DS) - The GC must be equipped for temperature programming, and all required accessories must be available, such as syringes, gases, and capillary columns.

4.1.1 GC Injection Port - The GC injection port must be designed for capillary columns. The use of splitless injection techniques is recommended. On column 1 µL injections can be used on the 60 m DB-5 column. The use of a moving needle injection port is also acceptable. When using the method described in this protocol, a 2 µL injection volume is used consistently (i.e., the injection volumes for all extracts, blanks, calibration solutions and the performance check samples are 2 µL). One µL injections are allowed; however, laboratories must remain

consistent throughout the analyses by using the same injection volume at all times.

4.1.2 Gas Chromatograph/Mass Spectrometer (GC/MS) Interface - The GC/MS interface components should withstand 350°C. The interface must be designed so that the separation of 2,3,7,8-TCDD from the other TCDD isomers achieved in the gas chromatographic column is not appreciably degraded. Cold spots or active surfaces (adsorption sites) in the GC/MS interface can cause peak tailing and peak broadening. It is recommended that the GC column be fitted directly into the mass spectrometer ion source without being exposed to the ionizing electron beam. Graphite ferrules should be avoided in the injection port because they may adsorb the PCDDs and PCDFs. Vespel™, or equivalent, ferrules are recommended.

4.1.3 Mass Spectrometer - The static resolving power of the instrument must be maintained at a minimum of 10,000 (10 percent valley).

4.1.4 Data System - A dedicated data system is employed to control the rapid multiple-ion monitoring process and to acquire the data. Quantitation data (peak areas or peak heights) and SIM traces (displays of intensities of each ion signal being monitored including the lock-mass ion as a function of time) must be acquired during the analyses and stored. Quantitations may be reported based upon computer generated peak areas or upon measured peak heights (chart recording). The data system must be capable of acquiring data at a minimum of 10 ions in a single scan. It is also recommended to have a data system capable of switching to different sets of ions (descriptors) at specified times during an HRGC/HRMS acquisition. The data system should be able to provide hard copies of individual ion chromatograms for selected gas chromatographic time intervals. It should also be able to acquire mass spectral peak profiles (Sec. 8.1.2.3) and provide hard copies of peak profiles to demonstrate the required resolving power. The data system should permit the measurement of noise on the base line.

NOTE: The detector ADC zero setting must allow peak-to-peak measurement of the noise on the base line of every monitored channel and allow for good estimation of the instrument resolving power. In Figure 2, the effect of different zero settings on the measured resolving power is shown.

4.2 GC Columns

4.2.1 In order to have an isomer specific determination for 2,3,7,8-TCDD and to allow the detection of OCDD/OCDF within a reasonable time interval in one HRGC/HRMS analysis, use of the 60 m DB-5 fused silica capillary column is recommended. Minimum acceptance criteria must be demonstrated and documented (Sec. 8.2.2). At the beginning of each 12 hour period (after mass resolution and GC resolution are demonstrated) during which sample extracts or concentration calibration solutions will be analyzed, column operating conditions must be attained for the required separation on the column to be used for samples. Operating conditions known to produce acceptable results with the recommended column are shown in Sec. 7.6.

4.2.2 Isomer specificity for all 2,3,7,8-substituted PCDDs/PCDFs cannot be achieved on the 60 m DB-5 GC column alone. In order to determine the proper concentrations of the individual 2,3,7,8-substituted congeners, the sample extract must be reanalyzed on another GC column that resolves the isomers.

4.2.3 30 m DB-225 fused silica capillary column, (J&W Scientific) or equivalent.

4.3 Miscellaneous Equipment and Materials - The following list of items does not necessarily constitute an exhaustive compendium of the equipment needed for this analytical method.

4.3.1 Nitrogen evaporation apparatus with variable flow rate.

4.3.2 Balances capable of accurately weighing to 0.01 g and 0.0001 g.

4.3.3 Centrifuge.

4.3.4 Water bath, equipped with concentric ring covers and capable of being temperature controlled within $\pm$ 2°C.

4.3.5 Stainless steel or glass container large enough to hold contents of one pint sample containers.

4.3.6 Glove box.

4.3.7 Drying oven.

4.3.8 Stainless steel spoons and spatulas.

4.3.9 Laboratory hoods.

4.3.10 Pipets, disposable, Pasteur, 150 mm long x 5 mm ID.

4.3.11 Pipets, disposable, serological, 10 mL, for the preparation of the carbon columns specified in Sec. 7.5.3.

4.3.12 Reaction vial, 2 mL, silanized amber glass (Reacti-vial, or equivalent).

4.3.13 Stainless steel meat grinder with a 3 to 5 mm hole size inner plate.

4.3.14 Separatory funnels, 125 mL and 2000 mL.

4.3.15 Kuderna-Danish concentrator, 500 mL, fitted with 10 mL concentrator tube and three ball Snyder column.

4.3.16 Teflon™ or carborundum (silicon carbide) boiling chips (or equivalent), washed with hexane before use.

NOTE: Teflon™ boiling chips may float in methylene chloride, may not work in the presence of any water phase, and may be penetrated by nonpolar organic compounds.

4.3.17 Chromatographic columns, glass, 300 mm x 10.5 mm, fitted with Teflon™ stopcock.

4.3.18 Adapters for concentrator tubes.

4.3.19 Glass fiber filters, 0.70 µm, Whatman GFF, or equivalent.

4.3.20 Dean-Stark trap, 5 or 10 mL, with T-joints, condenser and 125 mL flask.

4.3.21 Continuous liquid-liquid extractor.

4.3.22 All glass Soxhlet apparatus, 500 mL flask.

4.3.23 Soxhlet/Dean Stark extractor (optional), all glass, 500 mL flask.

4.3.24 Glass funnels, sized to hold 170 mL of liquid.

4.3.25 Desiccator.

4.3.26 Solvent reservoir (125 mL), Kontes; 12.35 cm diameter (special order item), compatible with gravity carbon column.

4.3.27 Rotary evaporator with a temperature controlled water bath.

4.3.28 High speed tissue homogenizer, equipped with an EN-8 probe, or equivalent.

4.3.29 Glass wool, extracted with methylene chloride, dried and stored in a clean glass jar.

4.3.30 Extraction jars, glass, 250 mL, with teflon lined screw cap.

4.3.31 Volumetric flasks, Class A - 10 mL to 1000 mL.

4.3.32 Glass vials, 1 dram (or metric equivalent).

NOTE: Reuse of glassware should be minimized to avoid the risk of contamination. All glassware that is reused must be scrupulously cleaned as soon as possible after use, according to the following procedure: Rinse glassware with the last solvent used in it. Wash with hot detergent water, then rinse with copious amounts of tap water and several portions of organic-free reagent water. Rinse with high purity acetone

and hexane and store it inverted or capped with solvent rinsed aluminum foil in a clean environment.

5.0 REAGENTS AND STANDARD SOLUTIONS

5.1 Organic-free reagent water - All references to water in this method refer to organic-free reagent water, as defined in Chapter One.

5.2 Column Chromatography Reagents

5.2.1 Alumina, neutral, 80/200 mesh (Super 1, Woelm®, or equivalent). Store in a sealed container at room temperature, in a desiccator, over self-indicating silica gel.

5.2.2 Alumina, acidic AG4, (Bio Rad Laboratories catalog #132-1240, or equivalent). Soxhlet extract with methylene chloride for 24 hours if blanks show contamination, and activate by heating in a foil covered glass container for 24 hours at 190°C. Store in a glass bottle sealed with a Teflon™ lined screw cap.

5.2.3 Silica gel, high purity grade, type 60, 70-230 mesh; Soxhlet extract with methylene chloride for 24 hours if blanks show contamination, and activate by heating in a foil covered glass container for 24 hours at 190°C. Store in a glass bottle sealed with a Teflon™ lined screw cap.

5.2.4 Silica gel impregnated with sodium hydroxide. Add one part (by weight) of 1 M NaOH solution to two parts (by weight) silica gel (extracted and activated) in a screw cap bottle and mix with a glass rod until free of lumps. Store in a glass bottle sealed with a Teflon™ lined screw cap.

5.2.5 Silica gel impregnated with 40 percent (by weight) sulfuric acid. Add two parts (by weight) concentrated sulfuric acid to three parts (by weight) silica gel (extracted and activated), mix with a glass rod until free of lumps, and store in a screw capped glass bottle. Store in a glass bottle sealed with a Teflon™ lined screw cap.

5.2.6 Celite 545® (Supelco), or equivalent.

5.2.7 Active carbon AX-21 (Anderson Development Co., Adrian, MI), or equivalent, prewashed with methanol and dried in vacuo at 110°C. Store in a glass bottle sealed with a Teflon™ lined screw cap.

5.3 Reagents

5.3.1 Sulfuric acid, H_2SO_4, concentrated, ACS grade, specific gravity 1.84.

5.3.2 Potassium hydroxide, KOH, ACS grade, 20 percent (w/v) in organic-free reagent water.

5.3.3 Sodium chloride, NaCl, analytical reagent, 5 percent (w/v) in organic-free reagent water.

5.3.4 Potassium carbonate, K_2CO_3, anhydrous, analytical reagent.

5.4 Desiccating agent

5.4.1 Sodium sulfate (powder, anhydrous), Na_2SO_4. Purify by heating at 400°C for 4 hours in a shallow tray, or by precleaning the sodium sulfate with methylene chloride. If the sodium sulfate is precleaned with methylene chloride, a method blank must be analyzed, demonstrating that there is no interference from the sodium sulfate.

5.5 Solvents

5.5.1 Methylene chloride, CH_2Cl_2. High purity, distilled in glass or highest available purity.

5.5.2 Hexane, C_6H_{14}. High purity, distilled in glass or highest available purity.

5.5.3 Methanol, CH_3OH. High purity, distilled in glass or highest available purity.

5.5.4 Nonane, C_9H_{20}. High purity, distilled in glass or highest available purity.

5.5.5 Toluene, $C_6H_5CH_3$. High purity, distilled in glass or highest available purity.

5.5.6 Cyclohexane, C_6H_{12}. High purity, distilled in glass or highest available purity.

5.5.7 Acetone, CH_3COCH_3. High purity, distilled in glass or highest available purity.

5.6 High-Resolution Concentration Calibration Solutions (Table 5) - Five nonane solutions containing unlabeled (totaling 17) and carbon-labeled (totaling 11) PCDDs and PCDFs at known concentrations are used to calibrate the instrument. The concentration ranges are homologue dependent, with the lowest values for the tetrachlorinated dioxin and furan (1.0 pg/µL) and the highest values for the octachlorinated congeners (1000 pg/µL).

5.6.1 Depending on the availability of materials, these high-resolution concentration calibration solutions may be obtained from the Environmental Monitoring Systems Laboratory, U.S. EPA, Cincinnati, Ohio. However, additional secondary standards must be obtained from commercial sources, and solutions should be prepared in the analyst's laboratory. It is the responsibility of the laboratory to ascertain that the calibration solutions received (or prepared) are indeed at the appropriate concentrations before they are used to analyze samples.

5.6.2 Store the concentration calibration solutions in 1 mL minivials at room temperature in the dark.

5.7 GC Column Performance Check Solution - This solution contains the first and last eluting isomers for each homologous series from tetra- through heptachlorinated congeners. The solution also contains a series of other TCDD isomers for the purpose of documenting the chromatographic resolution. The $^{13}C_{12}$-2,3,7,8-TCDD is also present. The laboratory is required to use nonane as the solvent and adjust the volume so that the final concentration does not exceed 100 pg/µL per congener. Table 7 summarizes the qualitative composition (minimum requirement) of this performance evaluation solution.

5.8 Sample Fortification Solution - This nonane solution contains the nine internal standards at the nominal concentrations that are listed in Table 2. The solution contains at least one carbon-labeled standard for each homologous series, and it is used to measure the concentrations of the native substances. (Note that $^{13}C_{12}$-OCDF is not present in the solution.)

5.9 Recovery Standard Solution - This nonane solution contains two recovery standards, $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD, at a nominal concentration of 50 pg/µL per compound. 10 to 50 µL of this solution will be spiked into each sample extract before the final concentration step and HRGC/HRMS analysis.

5.10 Matrix Spike Fortification Solution - Solution used to prepare the MS and MSD samples. It contains all unlabeled analytes listed in Table 5 at concentrations corresponding to the HRCC 3.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 See the introductory material to this chapter, Organic Analytes, Sec. 4.1.

6.2 Sample Collection

6.2.1 Sample collection personnel should, to the extent possible, homogenize samples in the field before filling the sample containers. This should minimize or eliminate the necessity for sample homogenization in the laboratory. The analyst should make a judgment, based on the appearance of the sample, regarding the necessity for additional mixing. If the sample is clearly not homogeneous, the entire contents should be transferred to a glass or stainless steel pan for mixing with a stainless steel spoon or spatula before removal of a sample portion for analysis.

6.2.2 Grab and composite samples must be collected in glass containers. Conventional sampling practices must be followed. The bottle must not be prewashed with sample before collection. Sampling equipment must be free of potential sources of contamination.

6.3 Grinding or Blending of Fish Samples - If not otherwise specified by the U.S. EPA, the whole fish (frozen) should be blended or ground to provide a homogeneous sample. The use of a stainless steel meat grinder with a 3 to 5 mm

hole size inner plate is recommended. In some circumstances, analysis of fillet or specific organs of fish may be requested by the U.S. EPA. If so requested, the above whole fish requirement is superseded.

6.4 Storage and Holding Times - All samples, except fish and adipose tissue samples, must be stored at 4°C in the dark, extracted within 30 days and completely analyzed within 45 days of extraction. Fish and adipose tissue samples must be stored at -20°C in the dark, extracted within 30 days and completely analyzed within 45 days of collection. Whenever samples are analyzed after the holding time expiration date, the results should be considered to be minimum concentrations and should be identified as such.

NOTE: The holding times listed in Sec. 6.4 are recommendations. PCDDs and PCDFs are very stable in a variety of matrices, and holding times under the conditions listed in Sec. 6.4 may be as high as a year for certain matrices. Sample extracts, however, should always be analyzed within 45 days of extraction.

6.5 Phase Separation - This is a guideline for phase separation for very wet (>25 percent water) soil, sediment and paper pulp samples. Place a 50 g portion in a suitable centrifuge bottle and centrifuge for 30 minutes at 2,000 rpm. Remove the bottle and mark the interface level on the bottle. Estimate the relative volume of each phase. With a disposable pipet, transfer the liquid layer into a clean bottle. Mix the solid with a stainless steel spatula and remove a portion to be weighed and analyzed (percent dry weight determination, extraction). Return the remaining solid portion to the original sample bottle (empty) or to a clean sample bottle that is properly labeled, and store it as appropriate. Analyze the solid phase by using only the soil, sediment and paper pulp method. Take note of, and report, the estimated volume of liquid before disposing of the liquid as a liquid waste.

6.6 Soil, Sediment, or Paper Sludge (Pulp) Percent Dry Weight Determination - The percent dry weight of soil, sediment or paper pulp samples showing detectable levels (see note below) of at least one 2,3,7,8-substituted PCDD/PCDF congener is determined according to the following procedure. Weigh a 10 g portion of the soil or sediment sample (± 0.5 g) to three significant figures. Dry it to constant weight at 110°C in an adequately ventilated oven. Allow the sample to cool in a desiccator. Weigh the dried solid to three significant figures. Calculate and report the percent dry weight. Do not use this solid portion of the sample for extraction, but instead dispose of it as hazardous waste.

NOTE: Until detection limits have been established (Sec. 1.3), the lower MCLs (Table 1) may be used to estimate the minimum detectable levels.

$$\% \text{ dry weight} = \frac{\text{g of dry sample}}{\text{g of sample}} \times 100$$

CAUTION: Finely divided soils and sediments contaminated with PCDDs/PCDFs are hazardous because of the potential for inhalation or ingestion of particles containing PCDDs/PCDFs

(including 2,3,7,8-TCDD). Such samples should be handled in a confined environment (i.e., a closed hood or a glove box).

6.7 Lipid Content Determination

6.7.1 Fish Tissue - To determine the lipid content of fish tissue, concentrate 125 mL of the fish tissue extract (Sec. 7.2.2), in a tared 200 mL round bottom flask, on a rotary evaporator until a constant weight (W) is achieved.

$$\text{Percent lipid} = \frac{100\ (W)}{10}$$

Dispose of the lipid residue as a hazardous waste if the results of the analysis indicate the presence of PCDDs or PCDFs.

6.7.2 Adipose Tissue - Details for the determination of the adipose tissue lipid content are provided in Sec. 7.3.3.

7.0 PROCEDURE

7.1 Internal standard addition

7.1.1 Use a portion of 1 g to 1000 g (± 5 percent) of the sample to be analyzed. Typical sample size requirements for different matrices are given in Sec. 7.4 and in Table 1. Transfer the sample portion to a tared flask and determine its weight.

7.1.2 Except for adipose tissue, add an appropriate quantity of the sample fortification mixture (Sec. 5.8) to the sample. All samples should be spiked with 100 µL of the sample fortification mixture to give internal standard concentrations as indicated in Table 1. As an example, for $^{13}C_{12}$-2,3,7,8-TCDD, a 10 g soil sample requires the addition of 1000 pg of $^{13}C_{12}$-2,3,7,8-TCDD to give the required 100 ppt fortification level. The fish tissue sample (20 g) must be spiked with 200 µL of the internal standard solution, because half of the extract will be used to determine the lipid content (Sec. 6.7.1).

7.1.2.1 For the fortification of soil, sediment, fly ash, water, fish tissue, paper pulp and wet sludge samples, mix the sample fortification solution with 1.0 mL acetone.

7.1.2.2 Do not dilute the nonane solution for the other matrices.

7.1.2.3 The fortification of adipose tissue is carried out at the time of homogenization (Sec. 7.3.2.3).

7.2 Extraction and Purification of Fish and Paper Pulp Samples

7.2.1 Add 60 g anhydrous sodium sulfate to a 20 g portion of a homogeneous fish sample (Sec. 6.3) and mix thoroughly with a stainless

steel spatula. After breaking up any lumps, place the fish/sodium sulfate mixture in the Soxhlet apparatus on top of a glass wool plug. Add 250 mL methylene chloride or hexane/methylene chloride (1:1) to the Soxhlet apparatus and reflux for 16 hours. The solvent must cycle completely through the system five times per hour. Follow the same procedure for the partially dewatered paper pulp sample (using a 10 g sample, 30 g of anhydrous sodium sulfate and 200 mL of toluene).

NOTE: As an option, a Soxhlet/Dean Stark extractor system may be used, with toluene as the solvent. No sodium sulfate is added when using this option.

7.2.2 Transfer the fish extract from Sec. 7.2.1 to a 250 mL volumetric flask and fill to the mark with methylene chloride. Mix well, then remove 125 mL for the determination of the lipid content (Sec. 6.7.1). Transfer the remaining 125 mL of the extract, plus two 15 mL hexane/methylene chloride rinses of the volumetric flask, to a KD apparatus equipped with a Snyder column. Quantitatively transfer all of the paper pulp extract to a KD apparatus equipped with a Snyder column.

NOTE: As an option, a rotary evaporator may be used in place of the KD apparatus for the concentration of the extracts.

7.2.3 Add a Teflon™, or equivalent, boiling chip. Concentrate the extract in a water bath to an apparent volume of 10 mL. Remove the apparatus from the water bath and allow to cool for 5 minutes.

7.2.4 Add 50 mL hexane and a new boiling chip to the KD flask. Concentrate in a water bath to an apparent volume of 5 mL. Remove the apparatus from the water bath and allow to cool for 5 minutes.

NOTE: The methylene chloride must have been completely removed before proceeding with the next step.

7.2.5 Remove and invert the Snyder column and rinse it into the KD apparatus with two 1 mL portions of hexane. Decant the contents of the KD apparatus and concentrator tube into a 125 mL separatory funnel. Rinse the KD apparatus with two additional 5 mL portions of hexane and add the rinses to the funnel. Proceed with the cleanup according to the instructions starting in Sec. 7.5.1.1, but omit the procedures described in Secs. 7.5.1.2 and 7.5.1.3.

7.3 Extraction and Purification of Human Adipose Tissue

7.3.1 Human adipose tissue samples must be stored at a temperature of -20°C or lower from the time of collection until the time of analysis. The use of chlorinated materials during the collection of the samples must be avoided. Samples are handled with stainless steel forceps, spatulas, or scissors. All sample bottles (glass) are cleaned as specified in the note at the end of Sec. 4.3. Teflon™ lined caps should be used.

NOTE: The specified storage temperature of -20°C is the maximum storage temperature permissible for adipose tissue samples. Lower storage temperatures are recommended.

7.3.2 Adipose Tissue Extraction

7.3.2.1 Weigh, to the nearest 0.01 g, a 10 g portion of a frozen adipose tissue sample into a culture tube (2.2 x 15 cm).

NOTE: The sample size may be smaller, depending on availability. In such a situation, the analyst is required to adjust the volume of the internal standard solution added to the sample to meet the fortification level stipulated in Table 1.

7.3.2.2 Allow the adipose tissue specimen to reach room temperature (up to 2 hours).

7.3.2.3 Add 10 mL methylene chloride and 100 µL of the sample fortification solution. Homogenize the mixture for approximately 1 minute with a tissue homogenizer.

7.3.2.4 Allow the mixture to separate, then remove the methylene chloride extract from the residual solid material with a disposable pipet. Percolate the methylene chloride through a filter funnel containing a clean glass wool plug and 10 g anhydrous sodium sulfate. Collect the dried extract in a graduated 100 mL volumetric flask.

7.3.2.5 Add a second 10 mL portion of methylene chloride to the sample and homogenize for 1 minute. Decant the solvent, dry it, and transfer it to the 100 mL volumetric flask (Sec. 7.3.2.4).

7.3.2.6 Rinse the culture tube with at least two additional portions of methylene chloride (10 mL each), and transfer the entire contents to the filter funnel containing the anhydrous sodium sulfate. Rinse the filter funnel and the anhydrous sodium sulfate contents with additional methylene chloride (20 to 40 mL) into the 100 mL flask. Discard the sodium sulfate.

7.3.2.7 Adjust the volume to the 100 mL mark with methylene chloride.

7.3.3 Adipose Tissue Lipid Content Determination

7.3.3.1 Preweigh a clean 1 dram (or metric equivalent) glass vial to the nearest 0.0001 g on an analytical balance tared to zero.

7.3.3.2 Accurately transfer 1.0 mL of the final extract (100 mL) from Sec. 7.3.2.7 to the vial. Reduce the volume of the extract on a water bath (50-60°C) by a gentle stream of purified

nitrogen until an oily residue remains. Nitrogen blowdown is continued until a constant weight is achieved.

NOTE: When the sample size of the adipose tissue is smaller than 10 g, then the analyst may use a larger portion (up to 10 percent) of the extract defined in Sec. 7.3.2.7 for the lipid determination.

7.3.3.3 Accurately weigh the vial with the residue to the nearest 0.0001 g and calculate the weight of the lipid present in the vial based on the difference of the weights.

7.3.3.4 Calculate the percent lipid content of the original sample to the nearest 0.1 percent as shown below:

$$\text{Lipid content, LC (\%)} = \frac{W_{lr} \times V_{ext}}{W_{at} \times V_{al}} \times 100$$

where:

W_{lr} = weight of the lipid residue to the nearest 0.0001 g calculated from Sec. 7.3.3.3,

V_{ext} = total volume (100 mL) of the extract in mL from Sec. 7.3.2.7,

W_{at} = weight of the original adipose tissue sample to the nearest 0.01 g from Sec. 7.3.2.1, and

V_{al} = volume of the aliquot of the final extract in mL used for the quantitative measure of the lipid residue (1.0 mL) from Sec. 7.3.3.2.

7.3.3.5 Record the lipid residue measured in Sec. 7.3.3.3 and the percent lipid content from Sec. 7.3.3.4.

7.3.4 Adipose Tissue Extract Concentration

7.3.4.1 Quantitatively transfer the remaining extract from Sec. 7.3.3.2 (99.0 mL) to a 500 mL Erlenmeyer flask. Rinse the volumetric flask with 20 to 30 mL of additional methylene chloride to ensure quantitative transfer.

7.3.4.2 Concentrate the extract on a rotary evaporator and a water bath at 40°C until an oily residue remains.

7.3.5 Adipose Tissue Extract Cleanup

7.3.5.1 Add 200 mL hexane to the lipid residue in the 500 mL Erlenmeyer flask and swirl the flask to dissolve the residue.

7.3.5.2 Slowly add, with stirring, 100 g of 40 percent (w/w) sulfuric acid-impregnated silica gel. Stir with a magnetic stirrer for two hours at room temperature.

7.3.5.3 Allow the solid phase to settle, and decant the liquid through a filter funnel containing 10 g anhydrous sodium sulfate on a glass wool plug, into another 500 mL Erlenmeyer flask.

7.3.5.4 Rinse the solid phase with two 50 mL portions of hexane. Stir each rinse for 15 minutes, decant, and dry as described under Sec. 7.3.5.3. Combine the hexane extracts from Sec. 7.3.5.3 with the rinses.

7.3.5.5 Rinse the sodium sulfate in the filter funnel with an additional 25 mL hexane and combine this rinse with the hexane extracts from Sec. 7.3.5.4.

7.3.5.6 Prepare an acidic silica column as follows: Pack a 2 cm x 10 cm chromatographic column with a glass wool plug, add approximately 20 mL hexane, add 1 g silica gel and allow to settle, then add 4 g of 40 percent (w/w) sulfuric acid-impregnated silica gel and allow to settle. Elute the excess hexane from the column until the solvent level reaches the top of the chromatographic packing. Verify that the column does not have any air bubbles and channels.

7.3.5.7 Quantitatively transfer the hexane extract from the Erlenmeyer flask (Secs. 7.3.5.3 through 7.3.5.5) to the silica gel column reservoir. Allow the hexane extract to percolate through the column and collect the eluate in a 500 mL KD apparatus.

7.3.5.8 Complete the elution by percolating 50 mL hexane through the column into the KD apparatus. Concentrate the eluate on a steam bath to approximately 5 mL. Use nitrogen blowdown to bring the final volume to about 100 µL.

NOTE: If the silica gel impregnated with 40 percent sulfuric acid is highly discolored throughout the length of the adsorbent bed, the cleaning procedure must be repeated beginning with Sec. 7.3.5.1.

7.3.5.9 The extract is ready for the column cleanups described in Secs. 7.5.2 through 7.5.3.6.

7.4 Extraction and Purification of Environmental and Waste Samples

7.4.1 Sludge/Wet Fuel Oil

7.4.1.1 Extract aqueous sludge or wet fuel oil samples by refluxing a sample (e.g., 2 g) with 50 mL toluene in a 125 mL flask fitted with a Dean-Stark water separator. Continue refluxing the sample until all the water is removed.

NOTE: If the sludge or fuel oil sample dissolves in toluene, treat it according to the instructions in Sec. 7.4.2 below. If the labeled sludge sample originates from pulp (paper mills), treat it according to the instructions starting in Sec. 7.2, but without the addition of sodium sulfate.

7.4.1.2 Cool the sample, filter the toluene extract through a glass fiber filter, or equivalent, into a 100 mL round bottom flask.

7.4.1.3 Rinse the filter with 10 mL toluene and combine the extract with the rinse.

7.4.1.4 Concentrate the combined solutions to near dryness on a rotary evaporator at 50°C. Use of an inert gas to concentrate the extract is also permitted. Proceed with Sec. 7.4.4.

7.4.2 Still Bottom/Oil

7.4.2.1 Extract still bottom or oil samples by mixing a sample portion (e.g., 1.0 g) with 10 mL toluene in a small beaker and filtering the solution through a glass fiber filter (or equivalent) into a 50 mL round bottom flask. Rinse the beaker and filter with 10 mL toluene.

7.4.2.2 Concentrate the combined toluene solutions to near dryness on a rotary evaporator at 50°C. Proceed with Sec. 7.4.4.

7.4.3 Fly Ash

NOTE: Because of the tendency of fly ash to "fly", all handling steps should be performed in a hood in order to minimize contamination.

7.4.3.1 Weigh about 10 g fly ash to two decimal places and transfer to an extraction jar. Add 100 µL sample fortification solution (Sec. 5.8), diluted to 1 mL with acetone, to the sample. Add 150 mL of 1 M HCl to the fly ash sample. Seal the jar with the Teflon™ lined screw cap and shake for 3 hours at room temperature.

7.4.3.2 Rinse a glass fiber filter with toluene, and filter the sample through the filter paper, placed in a Buchner funnel, into a 1 L flask. Wash the fly ash cake with approximately 500 mL organic-free reagent water and dry the filter cake overnight at room temperature in a desiccator.

7.4.3.3 Add 10 g anhydrous powdered sodium sulfate, mix thoroughly, let sit in a closed container for one hour, mix again, let sit for another hour, and mix again.

7.4.3.4 Place the sample and the filter paper into an extraction thimble, and extract in a Soxhlet extraction apparatus

charged with 200 mL toluene for 16 hours using a five cycle/hour schedule.

NOTE: As an option, a Soxhlet/Dean Stark extractor system may be used, with toluene as the solvent. No sodium sulfate is added when using this option.

7.4.3.5 Cool and filter the toluene extract through a glass fiber filter into a 500 mL round bottom flask. Rinse the filter with 10 mL toluene. Add the rinse to the extract and concentrate the combined toluene solutions to near dryness on a rotary evaporator at 50°C. Proceed with Sec. 7.4.4.

7.4.4 Transfer the concentrate to a 125 mL separatory funnel using 15 mL hexane. Rinse the flask with two 5 mL portions of hexane and add the rinses to the funnel. Shake the combined solutions in the separatory funnel for two minutes with 50 mL of 5 percent sodium chloride solution, discard the aqueous layer, and proceed with Sec. 7.5.

7.4.5 Aqueous samples

7.4.5.1 Allow the sample to come to ambient temperature, then mark the water meniscus on the side of the 1 L sample bottle for later determination of the exact sample volume. Add the required acetone diluted sample fortification solution (Sec. 5.8).

7.4.5.2 When the sample is judged to contain 1 percent or more solids, the sample must be filtered through a glass fiber filter that has been rinsed with toluene. If the suspended solids content is too great to filter through the 0.45 µm filter, centrifuge the sample, decant, and then filter the aqueous phase.

NOTE: Paper mill effluent samples normally contain 0.02%-0.2% solids, and would not require filtration. However, for optimum analytical results, all paper mill effluent samples should be filtered, the isolated solids and filtrate extracted separately, and the extracts recombined.

7.4.5.3 Combine the solids from the centrifuge bottle(s) with the particulates on the filter and with the filter itself and proceed with the Soxhlet extraction as specified in Secs. 7.4.6.1 through 7.4.6.4. Remove and invert the Snyder column and rinse it down into the KD apparatus with two 1 mL portions of hexane.

7.4.5.4 Pour the aqueous filtrate into a 2 L separatory funnel. Add 60 mL methylene chloride to the sample bottle, seal and shake for 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting.

7.4.5.5 Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface

between layers is more than one third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation (e.g., glass stirring rod).

7.4.5.6 Collect the methylene chloride into a KD apparatus (mounted with a 10 mL concentrator tube) by passing the sample extracts through a filter funnel packed with a glass wool plug and 5 g anhydrous sodium sulfate.

NOTE: As an option, a rotary evaporator may be used in place of the KD apparatus for the concentration of the extracts.

7.4.5.7 Repeat the extraction twice with fresh 60 mL portions of methylene chloride. After the third extraction, rinse the sodium sulfate with an additional 30 mL methylene chloride to ensure quantitative transfer. Combine all extracts and the rinse in the KD apparatus.

NOTE: A continuous liquid-liquid extractor may be used in place of a separatory funnel when experience with a sample from a given source indicates that a serious emulsion problem will result or an emulsion is encountered when using a separatory funnel. Add 60 mL methylene chloride to the sample bottle, seal, and shake for 30 seconds to rinse the inner surface. Transfer the solvent to the extractor. Repeat the rinse of the sample bottle with an additional 50 to 100 mL portion of methylene chloride and add the rinse to the extractor. Add 200 to 500 mL methylene chloride to the distilling flask, add sufficient organic-free reagent water (Sec. 5.1) to ensure proper operation, and extract for 24 hours. Allow to cool, then detach the distilling flask. Dry and concentrate the extract as described in Secs. 7.4.5.6 and 7.4.5.8 through 7.4.5.10. Proceed with Sec. 7.4.5.11.

7.4.5.8 Attach a Snyder column and concentrate the extract on a water bath until the apparent volume of the liquid is 5 mL. Remove the KD apparatus and allow it to drain and cool for at least 10 minutes.

7.4.5.9 Remove the Snyder column, add 50 mL hexane, add the concentrate obtained from the Soxhlet extraction of the suspended solids (Sec. 7.4.5.3), if applicable, re-attach the Snyder column, and concentrate to approximately 5 mL. Add a new boiling chip to the KD apparatus before proceeding with the second concentration step.

7.4.5.10 Rinse the flask and the lower joint with two 5 mL portions of hexane and combine the rinses with the extract to give a final volume of about 15 mL.

7.4.5.11 Determine the original sample volume by filling the sample bottle to the mark with water and transferring the water to a 1000 mL graduated cylinder. Record the sample volume to the nearest 5 mL. Proceed with Sec. 7.5.

7.4.6 Soil/Sediment

7.4.6.1 Add 10 g anhydrous powdered sodium sulfate to the sample portion (e.g., 10 g) and mix thoroughly with a stainless steel spatula. After breaking up any lumps, place the soil/sodium sulfate mixture in the Soxhlet apparatus on top of a glass wool plug (the use of an extraction thimble is optional).

NOTE: As an option, a Soxhlet/Dean Stark extractor system may be used, with toluene as the solvent. No sodium sulfate is added when using this option.

7.4.6.2 Add 200 to 250 mL toluene to the Soxhlet apparatus and reflux for 16 hours. The solvent must cycle completely through the system five times per hour.

NOTE: If the dried sample is not of free flowing consistency, more sodium sulfate must be added.

7.4.6.3 Cool and filter the extract through a glass fiber filter into a 500 mL round bottom flask for evaporation of the toluene. Rinse the filter with 10 mL of toluene, and concentrate the combined fractions to near dryness on a rotary evaporator at 50°C. Remove the flask from the water bath and allow to cool for 5 minutes.

7.4.6.4 Transfer the residue to a 125 mL separatory funnel, using 15 mL of hexane. Rinse the flask with two additional portions of hexane, and add the rinses to the funnel. Proceed with Sec. 7.5.

7.5 Cleanup

7.5.1 Partition

7.5.1.1 Partition the hexane extract against 40 mL of concentrated sulfuric acid. Shake for two minutes. Remove and discard the sulfuric acid layer (bottom). Repeat the acid washing until no color is visible in the acid layer (perform a maximum of four acid washings).

7.5.1.2 Omit this step for the fish sample extract. Partition the extract against 40 mL of 5 percent (w/v) aqueous sodium chloride. Shake for two minutes. Remove and discard the aqueous layer (bottom).

7.5.1.3 Omit this step for the fish sample extract. Partition the extract against 40 mL of 20 percent (w/v) aqueous

potassium hydroxide (KOH). Shake for two minutes. Remove and discard the aqueous layer (bottom). Repeat the base washing until no color is visible in the bottom layer (perform a maximum of four base washings). Strong base (KOH) is known to degrade certain PCDDs/PCDFs, so contact time must be minimized.

7.5.1.4 Partition the extract against 40 mL of 5 percent (w/v) aqueous sodium chloride. Shake for two minutes. Remove and discard the aqueous layer (bottom). Dry the extract by pouring it through a filter funnel containing anhydrous sodium sulfate on a glass wool plug, and collect it in a 50 mL round bottom flask. Rinse the funnel with the sodium sulfate with two 15 mL portions of hexane, add the rinses to the 50 mL flask, and concentrate the hexane solution to near dryness on a rotary evaporator (35°C water bath), making sure all traces of toluene (when applicable) are removed. (Use of blowdown with an inert gas to concentrate the extract is also permitted.)

7.5.2 Silica/Alumina Column Cleanup

7.5.2.1 Pack a gravity column (glass, 30 cm x 10.5 mm), fitted with a Teflon™ stopcock, with silica gel as follows: Insert a glass wool plug into the bottom of the column. Place 1 g silica gel in the column and tap the column gently to settle the silica gel. Add 2 g sodium hydroxide-impregnated silica gel, 4 g sulfuric acid-impregnated silica gel, and 2 g silica gel. Tap the column gently after each addition. A small positive pressure (5 psi) of clean nitrogen may be used if needed. Elute with 10 mL hexane and close the stopcock just before exposure of the top layer of silica gel to air. Discard the eluate. Check the column for channeling. If channeling is observed, discard the column. Do not tap the wetted column.

7.5.2.2 Pack a gravity column (glass, 300 mm x 10.5 mm), fitted with a Teflon™ stopcock, with alumina as follows: Insert a glass wool plug into the bottom of the column. Add a 4 g layer of sodium sulfate. Add a 4 g layer of Woelm® Super 1 neutral alumina. Tap the top of the column gently. Woelm® Super 1 neutral alumina need not be activated or cleaned before use, but it should be stored in a sealed desiccator. Add a 4 g layer of anhydrous sodium sulfate to cover the alumina. Elute with 10 mL hexane and close the stopcock just before exposure of the sodium sulfate layer to air. Discard the eluate. Check the column for channeling. If channeling is observed, discard the column. Do not tap a wetted column.

NOTE: Optionally, acidic alumina (Sec. 5.2.2) can be used in place of neutral alumina.

7.5.2.3 Dissolve the residue from Sec. 7.5.1.4 in 2 mL hexane and apply the hexane solution to the top of the silica gel column. Rinse the flask with enough hexane (3-4 mL) to complete the quantitative transfer of the sample to the surface of the silica gel.

7.5.2.4 Elute the silica gel column with 90 mL of hexane, concentrate the eluate on a rotary evaporator (35°C water bath) to approximately 1 mL, and apply the concentrate to the top of the alumina column (Sec. 7.5.2.2). Rinse the rotary evaporator flask twice with 2 mL of hexane, and add the rinses to the top of the alumina column.

7.5.2.5 Add 20 mL hexane to the alumina column and elute until the hexane level is just below the top of the sodium sulfate. Do not discard the eluted hexane, but collect it in a separate flask and store it for later use, as it may be useful in determining where the labeled analytes are being lost if recoveries are not satisfactory.

7.5.2.6 Add 15 mL of 60 percent methylene chloride in hexane (v/v) to the alumina column and collect the eluate in a conical shaped (15 mL) concentration tube. With a carefully regulated stream of nitrogen, concentrate the 60 percent methylene chloride/hexane fraction to about 2 mL.

7.5.3 Carbon Column Cleanup

7.5.3.1 Prepare an AX-21/Celite 545® column as follows: Thoroughly mix 5.40 g active carbon AX-21 and 62.0 g Celite 545® to produce an 8 percent (w/w) mixture. Activate the mixture at 130°C for 6 hours and store it in a desiccator.

7.5.3.2 Cut off both ends of a 10 mL disposable serological pipet to give a 10 cm long column. Fire polish both ends and flare, if desired. Insert a glass wool plug at one end, then pack the column with enough Celite 545® to form a 1 cm plug, add 1 g of the AX-21/Celite 545® mixture, top with additional Celite 545® (enough for a 1 cm plug), and cap the packing with another glass wool plug.

NOTE: Each new batch of AX-21/Celite 545® must be checked as follows: Add 50 µL of the continuing calibration solution to 950 µL hexane. Take this solution through the carbon column cleanup step, concentrate to 50 µL and analyze. If the recovery of any of the analytes is <80 percent, discard this batch of AX-21/Celite 545®.

7.5.3.3 Rinse the AX-21/Celite 545® column with 5 mL of toluene, followed by 2 mL of 75:20:5 (v/v) methylene chloride/methanol/toluene, 1 mL of 1:1 (v/v) cyclohexane/methylene chloride, and 5 mL hexane. The flow rate should be less than 0.5 mL/min. Discard the rinses. While the column is still wet with hexane, add the sample concentrate (Sec. 7.5.2.6) to the top of the column. Rinse the concentrator tube (which contained the sample concentrate) twice with 1 mL hexane, and add the rinses to the top of the column.

7.5.3.4 Elute the column sequentially with two 2 mL portions of hexane, 2 mL cyclohexane/methylene chloride (50:50,

v/v), and 2 mL methylene chloride/methanol/toluene (75:20:5, v/v). Combine these eluates; this combined fraction may be used as a check on column efficiency.

7.5.3.5 Turn the column upside down and elute the PCDD/PCDF fraction with 20 mL toluene. Verify that no carbon fines are present in the eluate. If carbon fines are present in the eluate, filter the eluate through a glass fiber filter (0.45 µm) and rinse the filter with 2 mL toluene. Add the rinse to the eluate.

7.5.3.6 Concentrate the toluene fraction to about 1 mL on a rotary evaporator by using a water bath at 50°C. Carefully transfer the concentrate into a 1 mL minivial and, again at elevated temperature (50°C), reduce the volume to about 100 µL using a stream of nitrogen and a sand bath. Rinse the rotary evaporator flask three times with 300 µL of a solution of 1 percent toluene in methylene chloride, and add the rinses to the concentrate. Add 10 µL of the nonane recovery standard solution (Sec. 5.9) for soil, sediment, water, fish, paper pulp and adipose tissue samples, or 50 µL of the recovery standard solution for sludge, still bottom and fly ash samples. Store the sample at room temperature in the dark.

7.6 Chromatographic/Mass Spectrometric Conditions and Data Acquisition Parameters

7.6.1 Gas Chromatograph

Column coating: DB-5
Film thickness: 0.25 µm
Column dimension: 60 m x 0.32 mm
Injector temperature: 270°C
Splitless valve time: 45 s
Interface temperature: Function of the final temperature
Temperature program:

Stage	Init. Temp. (°C)	Init. Hold Time (min)	Temp. Ramp (°C/min)	Final Temp. (°C)	Final Hold Time (min)
1	200	2	5	220	16
2			5	235	7
3			5	330	5

Total time: 60 min

7.6.2 Mass Spectrometer

7.6.2.1 The mass spectrometer must be operated in a selected ion monitoring (SIM) mode with a total cycle time (including the voltage reset time) of one second or less (Sec. 7.6.3.1). At a minimum, the ions listed in Table 6 for each of the

five SIM descriptors must be monitored. Note that with the exception of the last descriptor (OCDD/OCDF), all descriptors contain 10 ions. The selection (Table 6) of the molecular ions M and M+2 for ^{13}C-HxCDF and ^{13}C-HpCDF rather than M+2 and M+4 (for consistency) was made to eliminate, even under high-resolution mass spectrometric conditions, interferences occurring in these two ion channels for samples containing high levels of native HxCDDs and HpCDDs. It is important to maintain the same set of ions for both calibration and sample extract analyses. The selection of the lock-mass ion is left to the performing laboratory.

NOTE: At the option of the analyst, the tetra- and pentachlorinated dioxins and furans can be combined into a single descriptor.

7.6.2.2 The recommended mass spectrometer tuning conditions are based on the groups of monitored ions shown in Table 6. By using a PFK molecular leak, tune the instrument to meet the minimum required resolving power of 10,000 (10 percent valley) at m/z 304.9824 (PFK) or any other reference signal close to m/z 303.9016 (from TCDF). By using peak matching conditions and the aforementioned PFK reference peak, verify that the exact mass of m/z 380.9760 (PFK) is within 5 ppm of the required value. Note that the selection of the low- and high-mass ions must be such that they provide the largest voltage jump performed in any of the five mass descriptors (Table 6).

7.6.3 Data Acquisition

7.6.3.1 The total cycle time for data acquisition must be $\leq$ 1 second. The total cycle time includes the sum of all the dwell times and voltage reset times.

7.6.3.2 Acquire SIM data for all the ions listed in the five descriptors of Table 6.

7.7 Calibration

7.7.1 Initial Calibration - Initial calibration is required before any samples are analyzed for PCDDs and PCDFs. Initial calibration is also required if any routine calibration (Sec. 7.7.3) does not meet the required criteria listed in Sec. 7.7.2.

7.7.1.1 All five high-resolution concentration calibration solutions listed in Table 5 must be used for the initial calibration.

7.7.1.2 Tune the instrument with PFK as described in Sec. 7.6.2.2.

7.7.1.3 Inject 2 µL of the GC column performance check solution (Sec. 5.7) and acquire SIM mass spectral data as described earlier in Sec. 7.6.2. The total cycle time must be $\leq$ 1 second. The laboratory must not perform any further analysis until it is demonstrated and documented that the criterion listed in Sec. 8.2.1 was met.

7.7.1.4 By using the same GC (Sec. 7.6.1) and MS (Sec. 7.6.2) conditions that produced acceptable results with the column performance check solution, analyze a 2 µL portion of each of the five concentration calibration solutions once with the following mass spectrometer operating parameters.

7.7.1.4.1 The ratio of integrated ion current for the ions appearing in Table 8 (homologous series quantitation ions) must be within the indicated control limits (set for each homologous series) for all unlabeled calibration standards in Table 5.

7.7.1.4.2 The ratio of integrated ion current for the ions belonging to the carbon-labeled internal and recovery standards (Table 5) must be within the control limits stipulated in Table 8.

NOTE: Secs. 7.7.1.4.1 and 7.7.1.4.2 require that 17 ion ratios from Sec. 7.7.1.4.1 and 11 ion ratios from Sec. 7.7.1.4.2 be within the specified control limits simultaneously in one run. It is the laboratory's responsibility to take corrective action if the ion abundance ratios are outside the limits.

7.7.1.4.3 For each selected ion current profile (SICP) and for each GC signal corresponding to the elution of a target analyte and of its labeled standards, the signal-to-noise ratio (S/N) must be better than or equal to 2.5. Measurement of S/N is required for any GC peak that has an apparent S/N of less than 5:1. The result of the calculation must appear on the SICP above the GC peak in question.

7.7.1.4.4 Referring to Table 9, calculate the 17 relative response factors (RF) for unlabeled target analytes [RF(n); n = 1 to 17] relative to their appropriate internal standards (Table 5) and the nine RFs for the labeled $^{13}C_{12}$ internal standards [RF(m); m = 18 to 26)] relative to the two recovery standards (Table 5) according to the following formulae:

$$RF_n = \frac{A_x \times Q_{is}}{Q_x \times A_{is}} \qquad RF_m = \frac{A_{is} \times Q_{rs}}{Q_{is} \times A_{rs}}$$

where:

A_x = sum of the integrated ion abundances of the quantitation ions (Tables 6 and 9) for unlabeled PCDDs/PCDFs,

A_{is} = sum of the integrated ion abundances of the quantitation ions (Tables 6 and 9) for the labeled internal standards,

A_{rs} = sum of the integrated ion abundances of the quantitation ions (Tables 6 and 9) for the labeled recovery standards,

Q_{is} = quantity of the internal standard injected (pg),

Q_{rs} = quantity of the recovery standard injected (pg), and

Q_x = quantity of the unlabeled PCDD/PCDF analyte injected (pg).

The RF_n and RF_m are dimensionless quantities; the units used to express Q_{is}, Q_{rs} and Q_x must be the same.

7.7.1.4.5 Calculate the $\overline{RF}$ and their respective percent relative standard deviations (%RSD) for the five calibration solutions:

$$\overline{RF_n} = 1/5 \sum_{j=1}^{5} RF_{n(j)}$$

Where n represents a particular PCDD/PCDF (2,3,7,8-substituted) congener (n = 1 to 17; Table 9), and j is the injection number (or calibration solution number; j = 1 to 5).

7.7.1.4.6 The relative response factors to be used for the determination of the concentration of total isomers in a homologous series (Table 9) are calculated as follows:

7.7.1.4.6.1 For congeners that belong to a homologous series containing only one isomer (e.g., OCDD and OCDF) or only one 2,3,7,8-substituted isomer (Table 4; TCDD, PeCDD, HpCDD, and TCDF), the mean RF used will be the same as the mean RF determined in Sec. 7.7.1.4.5.

NOTE: The calibration solutions do not contain $^{13}C_{12}$-OCDF as an internal standard. This is because a minimum resolving power of 12,000 is required to resolve the $[M+6]^+$ ion of $^{13}C_{12}$-OCDF from the $[M+2]^+$ ion of OCDD (and $[M+4]^+$ from $^{13}C_{12}$-OCDF with $[M]^+$ of OCDD). Therefore, the RF for OCDF is calculated relative to $^{13}C_{12}$-OCDD.

7.7.1.4.6.2 For congeners that belong to a homologous series containing more than one 2,3,7,8-substituted isomer (Table 4), the mean RF used for those homologous series will be the mean of the RFs calculated for all individual 2,3,7,8-substituted congeners using the equation below:

$$\overline{RF_k} = \frac{1}{t} \sum_{n=1}^{t} RF_n$$

where:

k = 27 to 30 (Table 9), with 27 = PeCDF; 28 = HxCDF; 29 = HxCDD; and 30 = HpCDF,

t = total number of 2,3,7,8-substituted isomers present in the calibration solutions (Table 5) for each homologous series (e.g., two for PeCDF, four for HxCDF, three for HxCDD, two for HpCDF).

NOTE: Presumably, the HRGC/HRMS response factors of different isomers within a homologous series are different. However, this analytical protocol will make the assumption that the HRGC/HRMS responses of all isomers in a homologous series that do not have the 2,3,7,8-substitution pattern are the same as the responses of one or more of the 2,3,7,8-substituted isomer(s) in that homologous series.

7.7.1.4.7 Relative response factors [$\overline{RF_m}$] to be used for the determination of the percent recoveries for the nine internal standards are calculated as follows:

$$RF_m = \frac{A_{is}^m \times Q_{rs}}{Q_{is}^m \times A_{rs}}$$

$$\overline{RF_m} = 1/5 \sum_{j=1}^{5} RF_{m(j)},$$

where:

m = 18 to 26 (congener type) and j = 1 to 5 (injection number),

A_{is}^m = sum of the integrated ion abundances of the quantitation ions (Tables 6 and 9) for a given internal standard (m = 18 to 26),

A_{rs} = sum of the integrated ion abundances of the quantitation ions (Tables 6 and 9) for the appropriate recovery standard (see Table 5, footnotes),

Q_{rs}, Q_{is}^m = quantities of, respectively, the recovery standard (rs) and a particular internal standard (is = m) injected (pg),

RF_m = relative response factor of a particular internal standard (m) relative to an appropriate recovery standard, as determined from one injection, and

$\overline{RF_m}$ = calculated mean relative response factor of a particular internal standard (m) relative to an appropriate recovery standard, as determined from the five initial calibration injections (j).

7.7.2 Criteria for Acceptable Calibration - The criteria listed below for acceptable calibration must be met before sample analyses are performed.

7.7.2.1 The percent relative standard deviations for the mean response factors [RF_n and RF_m] from the 17 unlabeled standards must not exceed ± 20 percent, and those for the nine labeled reference compounds must not exceed ± 30 percent.

7.7.2.2 The S/N for the GC signals present in every SICP (including the ones for the labeled standards) must be $\geq$ 10.

7.7.2.3 The ion abundance ratios (Table 8) must be within the specified control limits.

NOTE: If the criterion for acceptable calibration listed in Sec. 7.7.2.1 is met, the analyte-specific RF can then be considered independent of the analyte quantity for the calibration concentration range. The mean RFs will be used for all calculations until the routine calibration criteria (Sec. 7.7.4) are no longer met. At such time, new mean RFs will be calculated from a new set of injections of the calibration solutions.

7.7.3 Routine Calibration (Continuing Calibration Check) - Routine calibrations must be performed at the beginning of a 12-hour period after successful mass resolution and GC resolution performance checks. A routine calibration is also required at the end of a 12-hour shift.

7.7.3.1 Inject 2 µL of the concentration calibration solution HRCC-3 standard (Table 5). By using the same HRGC/HRMS conditions as used in Secs. 7.6.1 and 7.6.2, determine and document an acceptable calibration as provided in Sec. 7.7.4.

7.7.4 Criteria for Acceptable Routine Calibration - The following criteria must be met before further analysis is performed.

7.7.4.1 The measured RFs [RF_n for the unlabeled standards] obtained during the routine calibration runs must be within $\pm$ 20 percent of the mean values established during the initial calibration (Sec. 7.7.1.4.5).

7.7.4.2 The measured RFs [RF_m for the labeled standards] obtained during the routine calibration runs must be within $\pm$ 30 percent of the mean values established during the initial calibration (Sec. 7.7.1.4.7).

7.7.4.3 The ion abundance ratios (Table 8) must be within the allowed control limits.

7.7.4.4 If either one of the criteria in Secs. 7.7.4.1 and 7.7.4.2 is not satisfied, repeat one more time. If these criteria are still not satisfied, the entire routine calibration process (Sec. 7.7.1) must be reviewed. It is realized that it may not always be possible to achieve all RF criteria. For example, it has occurred that the RF criteria for $^{13}C_{12}$-HpCDD and $^{13}C_{12}$-OCDD were not met, however, the RF values for the corresponding unlabeled compounds were routinely within the criteria established in the method. In these cases, 24 of the 26 RF parameters have met the QC criteria, and the data quality for the unlabeled HpCDD and OCDD values were not compromised as a result of the calibration event.

In these situations, the analyst must assess the effect on overall data quality as required for the data quality objectives and decide on appropriate action. Corrective action would be in order, for example, if the compounds for which the RF criteria were not met included both the unlabeled and the corresponding internal standard compounds. If the ion abundance ratio criterion (Sec. 7.7.4.3) is not satisfied, refer to the note in Sec. 7.7.1.4.2 for resolution.

NOTE: An initial calibration must be carried out whenever the HRCC-3, the sample fortification, or the recovery standard solution is replaced by a new solution from a different lot.

7.8 Analysis

7.8.1 Remove the sample or blank extract (from Sec. 7.5.3.6) from storage. With a stream of dry, purified nitrogen, reduce the extract volume to 10 µL to 50 µL.

NOTE: A final volume of 20 µL or more should be used whenever possible. A 10 µL final volume is difficult to handle, and injection of 2 µL out of 10 µL leaves little sample for confirmations and repeat injections, and for archiving.

7.8.2 Inject a 2 µL aliquot of the extract into the GC, operated under the conditions that have been established to produce acceptable results with the performance check solution (Secs. 7.6.1 and 7.6.2).

7.8.3 Acquire SIM data according to Secs. 7.6.2 and 7.6.3. Use the same acquisition and mass spectrometer operating conditions previously used to determine the relative response factors (Secs. 7.7.1.4.4 through 7.7.1.4.7). Ions characteristic of polychlorinated diphenyl ethers are included in the descriptors listed in Table 6.

NOTE: The acquisition period must at least encompass the PCDD/PCDF overall retention time window previously determined (Sec. 8.2.1.3). Selected ion current profiles (SICP) for the lock-mass ions (one per mass descriptor) must also be recorded and included in the data package. These SICPs must be true representations of the evolution of the lock-mass ions amplitudes during the HRGC/HRMS run (see Sec. 8.2.2 for the proper level of reference compound to be metered into the ion chamber.) The analyst may be required to monitor a PFK ion, not as a lock-mass, but as a regular ion, in order to meet this requirement. It is recommended to examine the lock-mass ion SICP for obvious basic sensitivity and stability changes of the instrument during the GC/MS run that could affect the measurements [Tondeur et al., 1984, 1987]. Report any discrepancies in the case narrative.

7.8.4 Identification Criteria - For a gas chromatographic peak to be identified as a PCDD or PCDF, it must meet all of the following criteria:

7.8.4.1 Retention Times

7.8.4.1.1 For 2,3,7,8-substituted congeners, which have an isotopically labeled internal or recovery standard present in the sample extract (this represents a total of 10 congeners including OCDD; Tables 2 and 3), the retention time (RRT; at maximum peak height) of the sample components (i.e., the two ions used for quantitation purposes listed in Table 6) must be within -1 to +3 seconds of the isotopically labeled standard.

7.8.4.1.2 For 2,3,7,8-substituted compounds that do not have an isotopically labeled internal standard present in the sample extract (this represents a total of six congeners; Table 3), the retention time must fall within 0.005 retention time units of the relative retention times measured in the routine calibration. Identification of OCDF is based on its retention time relative to $^{13}C_{12}$-OCDD as determined from the daily routine calibration results.

7.8.4.1.3 For non-2,3,7,8-substituted compounds (tetra through octa; totaling 119 congeners), the retention time must be within the corresponding homologous retention time windows established by analyzing the column performance check solution (Sec. 8.1.3).

7.8.4.1.4 The ion current responses for both ions used for quantitative purposes (e.g., for TCDDs: m/z 319.8965 and 321.8936) must reach maximum simultaneously ($\pm$ 2 seconds).

7.8.4.1.5 The ion current responses for both ions used for the labeled standards (e.g., for $^{13}C_{12}$-TCDD: m/z 331.9368 and m/z 333.9339) must reach maximum simultaneously ($\pm$ 2 seconds).

NOTE: The analyst is required to verify the presence of 1,2,8,9-TCDD and 1,3,4,6,8-PeCDF (Sec. 8.1.3) in the SICPs of the daily performance checks. Should either one compound be missing, the analyst is required to take corrective action as it may indicate a potential problem with the ability to detect all the PCDDs/PCDFs.

7.8.4.2 Ion Abundance Ratios

7.8.4.2.1 The integrated ion currents for the two ions used for quantitation purposes must have a ratio between the lower and upper limits established for the homologous series

to which the peak is assigned. See Secs. 7.7.1.4.1 and 7.7.1.4.2 and Table 8 for details.

7.8.4.3 Signal-to-Noise Ratio

7.8.4.3.1 All ion current intensities must be $\geq$ 2.5 times noise level for positive identification of a PCDD/PCDF compound or a group of coeluting isomers. Figure 6 describes the procedure to be followed for the determination of the S/N.

7.8.4.4 Polychlorinated Diphenyl Ether Interferences

7.8.4.4.1 In addition to the above criteria, the identification of a GC peak as a PCDF can only be made if no signal having a S/N $\geq$ 2.5 is detected at the same retention time ($\pm$ 2 seconds) in the corresponding polychlorinated diphenyl ether (PCDPE, Table 6) channel.

7.9 Calculations

7.9.1 For gas chromatographic peaks that have met the criteria outlined in Secs. 7.8.4.1.1 through 7.8.4.3.1, calculate the concentration of the PCDD or PCDF compounds using the formula:

$$C_x = \frac{A_x \times Q_{is}}{A_{is} \times W \times \overline{RF_n}}$$

where:

C_x = concentration of unlabeled PCDD/PCDF congeners (or group of coeluting isomers within an homologous series) in pg/g,

A_x = sum of the integrated ion abundances of the quantitation ions (Table 6) for unlabeled PCDDs/PCDFs,

A_{is} = sum of the integrated ion abundances of the quantitation ions (Table 6) for the labeled internal standards,

Q_{is} = quantity, in pg, of the internal standard added to the sample before extraction,

W = weight, in g, of the sample (solid or organic liquid), or volume in mL of an aqueous sample, and

$\overline{RF_n}$ = calculated mean relative response factor for the analyte [RF_n with n = 1 to 17; Sec. 7.7.1.4.5].

If the analyte is identified as one of the 2,3,7,8-substituted PCDDs or PCDFs, RF_n is the value calculated using the equation in Sec. 7.7.1.4.5. However, if it is a non-2,3,7,8-substituted congener, the RF(k) value is

the one calculated using the equation in Sec. 7.7.1.4.6.2. [RF_k k = 27 to 30].

7.9.2 Calculate the percent recovery of the nine internal standards measured in the sample extract, using the formula:

$$\text{Internal standard percent recovery} = \frac{A_{is} \times Q_{rs}}{Q_{is} \times A_{rs} \times \overline{RF_m}} \times 100$$

where:

A_{is} = sum of the integrated ion abundances of the quantitation ions (Table 6) for the labeled internal standard,

A_{rs} = sum of the integrated ion abundances of the quantitation ions (Table 6) for the labeled recovery standard; the selection of the recovery standard depends on the type of congeners (see Table 5, footnotes),

Q_{is} = quantity, in pg, of the internal standard added to the sample before extraction,

Q_{rs} = quantity, in pg, of the recovery standard added to the cleaned-up sample residue before HRGC/HRMS analysis, and

$\overline{RF_m}$ = calculated mean relative response factor for the labeled internal standard relative to the appropriate (see Table 5, footnotes) recovery standard. This represents the mean obtained in Sec. 7.7.1.4.7 [RF_m with m = 18 to 26].

NOTE: For human adipose tissue, adjust the percent recoveries by adding 1 percent to the calculated value to compensate for the 1 percent of the extract diverted for the lipid determination.

7.9.3 If the concentration in the final extract of any of the fifteen 2,3,7,8-substituted PCDD/PCDF compounds (Table 3) exceeds the upper method calibration limits (MCL) listed in Table 1 (e.g., 200 pg/µL for TCDD in soil), the linear range of response versus concentration may have been exceeded, and a second analysis of the sample (using a one tenth aliquot) should be undertaken. The volumes of the internal and recovery standard solutions should remain the same as described for the sample preparation (Secs. 7.1 to 7.9.3). For the other congeners (including OCDD), however, report the measured concentration and indicate that the value exceeds the MCL.

7.9.3.1 If a smaller sample size would not be representative of the entire sample, one of the following options is recommended:

(1) Re-extract an additional aliquot of sufficient size to insure that it is representative of the entire sample. Spike it with a

higher concentration of internal standard. Prior to GC/MS analysis, dilute the sample so that it has a concentration of internal standard equivalent to that present in the calibration standard. Then, analyze the diluted extract.

(2) Re-extract an additional aliquot of sufficient size to insure that it is representative of the entire sample. Spike it with a higher concentration of internal standard. Immediately following extraction, transfer the sample to a volumetric flask and dilute to known volume. Remove an appropriate aliquot and proceed with cleanup and analysis.

(3) Use the original analysis data to quantitate the internal standard recoveries. Respike the original extract (note that no additional cleanup is necessary) with 100 times the usual quantity of internal standards. Dilute the re-spiked extract by a factor of 100. Reanalyze the diluted sample using the internal standard recoveries calculated from the initial analysis to correct the results for losses during isolation and cleanup.

7.9.4 The total concentration for each homologous series of PCDD and PCDF is calculated by summing up the concentrations of all positively identified isomers of each homologous series. Therefore, the total should also include the 2,3,7,8-substituted congeners. The total number of GC signals included in the homologous total concentration value must be specified in the report. If an isomer is not detected, use zero (0) in this calculation.

7.9.5 Sample Specific Estimated Detection Limit - The sample specific estimated detection limit (EDL) is the concentration of a given analyte required to produce a signal with a peak height of at least 2.5 times the background signal level. An EDL is calculated for each 2,3,7,8-substituted congener that is not identified, regardless of whether or not other non-2,3,7,8-substituted isomers are present. Two methods of calculation can be used, as follows, depending on the type of response produced during the analysis of a particular sample.

7.9.5.1 Samples giving a response for both quantitation ions (Tables 6 and 9) that is less than 2.5 times the background level.

7.9.5.1.1 Use the expression for EDL (specific 2,3,7,8-substituted PCDD/PCDF) below to calculate an EDL for each absent 2,3,7,8-substituted PCDD/PCDF (i.e., S/N < 2.5). The background level is determined by measuring the range of the noise (peak to peak) for the two quantitation ions (Table 6) of a particular 2,3,7,8-substituted isomer within an homologous series, in the region of the SICP trace corresponding to the elution of the internal standard (if the congener possesses an internal standard) or in the region of the SICP where the congener is expected to elute by comparison with the routine calibration data (for those congeners that do not have a ^{13}C-labeled standard),

multiplying that noise height by 2.5, and relating the product to an estimated concentration that would produce that peak height.

Use the formula:

$$\text{EDL (specific 2,3,7,8-subst. PCDD/PCDF)} = \frac{2.5 \times H_x \times Q_{is}}{H_{is} \times W \times \overline{RF_n}}$$

where:

EDL = estimated detection limit for homologous 2,3,7,8-substituted PCDDs/PCDFs.

H_x = sum of the height of the noise level for each quantitation ion (Table 6) for the unlabeled PCDDs/PCDFs, measured as shown in Figure 6.

H_{is} = sum of the height of the noise level for each quantitation ion (Table 6) for the labeled internal standard, measured as shown in Figure 6.

W, $\overline{RF_n}$, and Q_{is} retain the same meanings as defined in Sec. 7.9.1.

7.9.5.2 Samples characterized by a response above the background level with a S/N of at least 2.5 for both quantitation ions (Tables 6 and 9).

7.9.5.2.1 When the response of a signal having the same retention time as a 2,3,7,8-substituted congener has a S/N in excess of 2.5 and does not meet any of the other qualitative identification criteria listed in Sec. 7.8.4, calculate the "Estimated Maximum Possible Concentration" (EMPC) according to the expression shown in Sec. 7.9.1, except that A_x in Sec. 7.9.1 should represent the sum of the area under the smaller peak and of the other peak area calculated using the theoretical chlorine isotope ratio.

7.9.6 The relative percent difference (RPD) of any duplicate sample results are calculated as follows:

$$RPD = \frac{| S_1 - S_2 |}{(S_1 + S_2) / 2} \times 100$$

S_1 and S_2 represent sample and duplicate sample results.

7.9.7 The 2,3,7,8-TCDD toxicity equivalents (TE) of PCDDs and PCDFs present in the sample are calculated, if requested by the data user, according to the method recommended by the Chlorinated Dioxins Workgroup

(CDWG) of the EPA and the Center for Disease Control (CDC). This method assigns a 2,3,7,8-TCDD toxicity equivalency factor (TEF) to each of the fifteen 2,3,7,8-substituted PCDDs and PCDFs (Table 3) and to OCDD and OCDF, as shown in Table 10. The 2,3,7,8-TCDD equivalent of the PCDDs and PCDFs present in the sample is calculated by summing the TEF times their concentration for each of the compounds or groups of compounds listed in Table 10. The exclusion of other homologous series such as mono-, di-, and tri- chlorinated dibenzodioxins and dibenzofurans does not mean that they are non-toxic. However, their toxicity, as known at this time, is much lower than the toxicity of the compounds listed in Table 10. The above procedure for calculating the 2,3,7,8-TCDD toxicity equivalents is not claimed by the CDWG to be based on a thoroughly established scientific foundation. The procedure, rather, represents a "consensus recommendation on science policy". Since the procedure may be changed in the future, reporting requirements for PCDD and PCDF data would still include the reporting of the analyte concentrations of the PCDD/PCDF congener as calculated in Secs. 7.9.1 and 7.9.4.

7.9.7.1 Two GC Column TEF Determination

7.9.7.1.1 The concentration of 2,3,7,8-TCDD (see note below), is calculated from the analysis of the sample extract on the 60 m DB-5 fused silica capillary column. The experimental conditions remain the same as the conditions described previously in Sec. 7.8, and the calculations are performed as outlined in Sec. 7.9. The chromatographic separation between the 2,3,7,8-TCDD and its close eluters (1,2,3,7/1,2,3,8-TCDD and 1,2,3,9-TCDD) must be equal or less than 25 percent valley.

7.9.7.1.2 The concentration of the 2,3,7,8-TCDF is obtained from the analysis of the sample extract on the 30 m DB-225 fused silica capillary column. However, the GC/MS conditions must be altered so that: (1) only the first three descriptors (i.e., tetra-, penta-, and hexachlorinated congeners) of Table 6 are used; and (2) the switching time between descriptor 2 (pentachlorinated congeners) and descriptor 3 (hexachlorinated congeners) takes place following the elution of $^{13}C_{12}$-1,2,3,7,8-PeCDD. The concentration calculations are performed as outlined in Sec. 7.9. The chromatographic separation between the 2,3,7,8-TCDF and its close eluters (2,3,4,7-TCDF and 1,2,3,9-TCDF) must be equal or less than 25 percent valley.

NOTE: The confirmation and quantitation of 2,3,7,8-TCDD (Sec. 7.9.7.1.1) may be accomplished on the SP-2330 GC column instead of the DB-5 column, provided the criteria listed in Sec. 8.2.1 are met and the requirements described in Sec. 8.3.2 are followed.

7.9.7.1.3 For a gas chromatographic peak to be identified as a 2,3,7,8-substituted PCDD/PCDF congener, it must meet the ion abundance and signal-to-noise ratio criteria listed in Secs. 7.8.4.2 and 7.8.4.3, respectively. In addition, the retention time identification criterion described in Sec. 7.8.4.1.1 applies here for congeners for which a carbon-labeled analogue is available in the sample extract. However, the relative retention time (RRT) of the 2,3,7,8-substituted congeners for which no carbon-labeled analogues are available must fall within 0.006 units of the carbon-labeled standard RRT. Experimentally, this is accomplished by using the attributions described in Table 11 and the results from the routine calibration run on the SP-2330 column.

8.0 QUALITY CONTROL

8.1 Refer to Chapter One for specific quality control (QC) procedures. Quality control to validate sample extraction is covered in Method 3500. If extract cleanup was performed, follow the QC in Method 3600 and in the specific cleanup method.

8.2 System Performance Criteria - System performance criteria are presented below. The laboratory may use the recommended GC column described in Sec. 4.2. It must be documented that all applicable system performance criteria (specified in Secs. 8.2.1 and 8.2.2) were met before analysis of any sample is performed. Sec. 7.6.1 provides recommended GC conditions that can be used to satisfy the required criteria. Figure 3 provides a typical 12-hour analysis sequence, whereby the response factors and mass spectrometer resolving power checks must be performed at the beginning and the end of each 12-hour period of operation. A GC column performance check is only required at the beginning of each 12-hour period during which samples are analyzed. An HRGC/HRMS method blank run is required between a calibration run and the first sample run. The same method blank extract may thus be analyzed more than once if the number of samples within a batch requires more than 12 hours of analyses.

8.2.1 GC Column Performance

8.2.1.1 Inject 2 µL (Sec. 4.1.1) of the column performance check solution (Sec. 5.7) and acquire selected ion monitoring (SIM) data as described in Sec. 7.6.2 within a total cycle time of $\leq$ 1 second (Sec. 7.6.3.1).

8.2.1.2 The chromatographic separation between 2,3,7,8-TCDD and the peaks representing any other unlabeled TCDD isomers must be resolved with a valley of $\leq$ 25 percent (Figure 4), where:

Valley percent = (x/y) (100)

x = measured as in Figure 4 from the 2,3,7,8-closest TCDD eluting isomer, and
y = the peak height of 2,3,7,8-TCDD.

It is the responsibility of the laboratory to verify the conditions suitable for the appropriate resolution of 2,3,7,8-TCDD from all other TCDD isomers. The GC column performance check solution also contains the known first and last PCDD/PCDF eluters under the conditions specified in this protocol. Their retention times are used to determine the eight homologue retention time windows that are used for qualitative (Sec. 7.8.4.1) and quantitative purposes. All peaks (that includes $^{13}C_{12}$-2,3,7,8-TCDD) should be labeled and identified on the chromatograms. Furthermore, all first eluters of a homologous series should be labeled with the letter F, and all last eluters of a homologous series should be labeled with the letter L (Figure 4 shows an example of peak labeling for TCDD isomers). Any individual selected ion current profile (SICP) (for the tetras, this would be the SICP for m/z 322 and m/z 304) or the reconstructed homologue ion current (for the tetras, this would correspond to m/z 320 + m/z 322 + m/z 304 + m/z 306) constitutes an acceptable form of data presentation. An SICP for the labeled compounds (e.g., m/z 334 for labeled TCDD) is also required.

8.2.1.3 The retention times for the switching of SIM ions characteristic of one homologous series to the next higher homologous series must be indicated in the SICP. Accurate switching at the appropriate times is absolutely necessary for accurate monitoring of these compounds. Allowable tolerance on the daily verification with the GC performance check solution should be better than 10 seconds for the absolute retention times of all the components of the mixture. Particular caution should be exercised for the switching time between the last tetrachlorinated congener (i.e., 1,2,8,9-TCDD) and the first pentachlorinated congener (i.e., 1,3,4,6,8-PeCDF), as these two compounds elute within 15 seconds of each other on the 60 m DB-5 column. A laboratory with a GC/MS system that is not capable of detecting both congeners (1,2,8,9-TCDD and 1,3,4,6,8-PeCDF) within one analysis must take corrective action. If the recommended column is not used, then the first and last eluting isomer of each homologue must be determined experimentally on the column which is used, and the appropriate isomers must then be used for window definition and switching times.

8.2.2 Mass Spectrometer Performance

8.2.2.1 The mass spectrometer must be operated in the electron ionization mode. A static resolving power of at least 10,000 (10 percent valley definition) must be demonstrated at appropriate masses before any analysis is performed (Sec. 7.8). Static resolving power checks must be performed at the beginning and at the end of each 12 hour period of operation. However, it is recommended that a check of the static resolution be made and documented before and after each analysis. Corrective action must be implemented whenever the resolving power does not meet the requirement.

8.2.2.2 Chromatography time for PCDDs and PCDFs exceeds the long term mass stability of the mass spectrometer. Because the instrument is operated in the high-resolution mode, mass drifts of a few ppm (e.g., 5 ppm in mass) can have serious adverse effects on instrument performance. Therefore, a mass drift correction is mandatory. To that effect, it is recommended to select a lock-mass ion from the reference compound (PFK is recommended) used for tuning the mass spectrometer. The selection of the lock-mass ion is dependent on the masses of the ions monitored within each descriptor. Table 6 offers some suggestions for the lock-mass ions. However, an acceptable lock-mass ion at any mass between the lightest and heaviest ion in each descriptor can be used to monitor and correct mass drifts. The level of the reference compound (PFK) metered into the ion chamber during HRGC/HRMS analyses should be adjusted so that the amplitude of the most intense selected lock-mass ion signal (regardless of the descriptor number) does not exceed 10 percent of the full scale deflection for a given set of detector parameters. Under those conditions, sensitivity changes that might occur during the analysis can be more effectively monitored.

NOTE: Excessive PFK (or any other reference substance) may cause noise problems and contamination of the ion source resulting in an increase in downtime for source cleaning.

8.2.2.3 Documentation of the instrument resolving power must then be accomplished by recording the peak profile of the high-mass reference signal (m/z 380.9760) obtained during the above peak matching experiment by using the low-mass PFK ion at m/z 304.9824 as a reference. The minimum resolving power of 10,000 must be demonstrated on the high-mass ion while it is transmitted at a lower accelerating voltage than the low-mass reference ion, which is transmitted at full sensitivity. The format of the peak profile representation (Figure 5) must allow manual determination of the resolution, i.e., the horizontal axis must be a calibrated mass scale (amu or ppm per division). The result of the peak width measurement (performed at 5 percent of the maximum, which corresponds to the 10 percent valley definition) must appear on the hard copy and cannot exceed 100 ppm at m/z 380.9760 (or 0.038 amu at that particular mass).

8.3 Quality Control Samples

8.3.1 Performance Evaluation Samples - Included among the samples in all batches may be samples (blind or double blind) containing known amounts of unlabeled 2,3,7,8-substituted PCDDs/PCDFs or other PCDD/PCDF congeners.

8.3.2 Performance Check Solutions

8.3.2.1 At the beginning of each 12-hour period during which samples are to be analyzed, an aliquot of the 1) GC column performance check solution and 2) high-resolution concentration

calibration solution No. 3 (HRCC-3; see Table 5) shall be analyzed to demonstrate adequate GC resolution and sensitivity, response factor reproducibility, and mass range calibration, and to establish the PCDD/PCDF retention time windows. A mass resolution check shall also be performed to demonstrate adequate mass resolution using an appropriate reference compound (PFK is recommended). If the required criteria are not met, remedial action must be taken before any samples are analyzed.

8.3.2.2 To validate positive sample data, the routine or continuing calibration (HRCC-3; Table 5) and the mass resolution check must be performed also at the end of each 12-hour period during which samples are analyzed. Furthermore, an HRGC/HRMS method blank run must be recorded following a calibration run and the first sample run.

8.3.2.2.1 If the laboratory operates only during one period (shift) each day of 12 hours or less, the GC performance check solution must be analyzed only once (at the beginning of the period) to validate the data acquired during the period. However, the mass resolution and continuing calibration checks must be performed at the beginning as well as at the end of the period.

8.3.2.2.2 If the laboratory operates during consecutive 12-hour periods (shifts), analysis of the GC performance check solution must be performed at the beginning of each 12-hour period. The mass resolution and continuing calibration checks from the previous period can be used for the beginning of the next period.

8.3.2.3 Results of at least one analysis of the GC column performance check solution and of two mass resolution and continuing calibration checks must be reported with the sample data collected during a 12 hour period.

8.3.2.4 Deviations from criteria specified for the GC performance check or for the mass resolution check invalidate all positive sample data collected between analyses of the performance check solution, and the extracts from those positive samples shall be reanalyzed.

If the routine calibration run fails at the beginning of a 12 hour shift, the instructions in Sec. 7.7.4.4 must be followed. If the continuing calibration check performed at the end of a 12 hour period fails by no more than 25 percent RPD for the 17 unlabeled compounds and 35 percent RPD for the 9 labeled reference compounds, use the mean RFs from the two daily routine calibration runs to compute the analyte concentrations, instead of the RFs obtained from the initial calibration. A new initial calibration (new RFs) is required immediately (within two hours) following the analysis of the samples, whenever the RPD from the end-of-shift routine calibration exceeds 25 percent or 35 percent, respectively. Failure

to perform a new initial calibration immediately following the analysis of the samples will automatically require reanalysis of all positive sample extracts analyzed before the failed end-of-shift continuing calibration check.

8.3.3 The GC column performance check mixture, high-resolution concentration calibration solutions, and the sample fortification solutions may be obtained from the EMSL-CIN. However, if not available from the EMSL-CIN, standards can be obtained from other sources, and solutions can be prepared in the laboratory. Concentrations of all solutions containing 2,3,7,8-substituted PCDDs/PCDFs, which are not obtained from the EMSL-CIN, must be verified by comparison with the EPA standard solutions that are available from the EMSL-CIN.

8.3.4 Field Blanks - Each batch of samples usually contains a field blank sample of uncontaminated soil, sediment or water that is to be fortified before analysis according to Sec. 8.3.4.1. In addition to this field blank, a batch of samples may include a rinsate, which is a portion of the solvent (usually trichloroethylene) that was used to rinse sampling equipment. The rinsate is analyzed to assure that the samples were not contaminated by the sampling equipment.

8.3.4.1 Fortified Field Blank

8.3.4.1.1 Weigh a 10 g portion or use 1 L (for aqueous samples) of the specified field blank sample and add 100 µL of the solution containing the nine internal standards (Table 2) diluted with 1.0 mL acetone (Sec. 7.1).

8.3.4.1.2 Extract by using the procedures beginning in Secs. 7.4.5 or 7.4.6, as applicable, add 10 µL of the recovery standard solution (Sec. 7.5.3.6) and analyze a 2 µL aliquot of the concentrated extract.

8.3.4.1.3 Calculate the concentration (Sec. 7.9.1) of 2,3,7,8-substituted PCDDs/PCDFs and the percent recovery of the internal standards (Sec. 7.9.2).

8.3.4.1.4 Extract and analyze a new simulated fortified field blank whenever new lots of solvents or reagents are used for sample extraction or for column chromatographic procedures.

8.3.4.2 Rinsate Sample

8.3.4.2.1 The rinsate sample must be fortified like a regular sample.

8.3.4.2.2 Take a 100 mL ($\pm$ 0.5 mL) portion of the sampling equipment rinse solvent (rinsate sample), filter, if necessary, and add 100 µL of the solution containing the nine internal standards (Table 2).

8.3.4.2.3 Using a KD apparatus, concentrate to approximately 5 mL.

NOTE: As an option, a rotary evaporator may be used in place of the KD apparatus for the concentration of the rinsate.

8.3.4.2.4 Transfer the 5 mL concentrate from the KD concentrator tube in 1 mL portions to a 1 mL minivial, reducing the volume in the minivial as necessary with a gentle stream of dry nitrogen.

8.3.4.2.5 Rinse the KD concentrator tube with two 0.5 mL portions of hexane and transfer the rinses to the 1 mL minivial. Blow down with dry nitrogen as necessary.

8.3.4.2.6 Just before analysis, add 10 µL recovery standard solution (Table 2) and reduce the volume to its final volume, as necessary (Sec. 7.8.1). No column chromatography is required.

8.3.4.2.7 Analyze an aliquot following the same procedures used to analyze samples.

8.3.4.2.8 Report percent recovery of the internal standard and the presence of any PCDD/PCDF compounds in µg/L of rinsate solvent.

8.3.5 Duplicate Analyses

8.3.5.1 In each batch of samples, locate the sample specified for duplicate analysis, and analyze a second 10 g soil or sediment sample portion or 1 L water sample, or an appropriate amount of the type of matrix under consideration.

8.3.5.1.1 The results of the laboratory duplicates (percent recovery and concentrations of 2,3,7,8-substituted PCDD/PCDF compounds) should agree within 25 percent relative difference (difference expressed as percentage of the mean). Report all results.

8.3.5.1.2 Recommended actions to help locate problems:

8.3.5.1.2.1 Verify satisfactory instrument performance (Secs. 8.2 and 8.3).

8.3.5.1.2.2 If possible, verify that no error was made while weighing the sample portions.

8.3.5.1.2.3 Review the analytical procedures with the performing laboratory personnel.

8.3.6 Matrix Spike and Matrix Spike Duplicate

8.3.6.1 Locate the sample for the MS and MSD analyses (the sample may be labeled "double volume").

8.3.6.2 Add an appropriate volume of the matrix spike fortification solution (Sec. 5.10) and of the sample fortification solution (Sec. 5.8), adjusting the fortification level as specified in Table 1 under IS Spiking Levels.

8.3.6.3 Analyze the MS and MSD samples as described in Sec. 7.

8.3.6.4 The results obtained from the MS and MSD samples (concentrations of 2,3,7,8-substituted PCDDs/PCDFs) should agree within 20 percent relative difference.

8.4 Percent Recovery of the Internal Standards - For each sample, method blank and rinsate, calculate the percent recovery (Sec. 7.9.2). The percent recovery should be between 40 percent and 135 percent for all 2,3,7,8-substituted internal standards.

NOTE: A low or high percent recovery for a blank does not require discarding the analytical data but it may indicate a potential problem with future analytical data.

8.5 Identification Criteria

8.5.1 If either one of the identification criteria appearing in Secs. 7.8.4.1.1 through 7.8.4.1.4 is not met for an homologous series, it is reported that the sample does not contain unlabeled 2,3,7,8-substituted PCDD/PCDF isomers for that homologous series at the calculated detection limit (Sec. 7.9.5)

8.5.2 If the first initial identification criteria (Secs. 7.8.4.1.1 through 7.8.4.1.4) are met, but the criteria appearing in Secs. 7.8.4.1.5 and 7.8.4.2.1 are not met, that sample is presumed to contain interfering contaminants. This must be noted on the analytical report form, and the sample should be rerun or the extract reanalyzed.

8.6 Unused portions of samples and sample extracts should be preserved for six months after sample receipt to allow further analyses.

8.7 Reuse of glassware is to be minimized to avoid the risk of contamination.

9.0 METHOD PERFORMANCE

9.1 Data are currently not available.

10.0 REFERENCES

1. "Control of Interferences in the Analysis of Human Adipose Tissue for 2,3,7,8-Tetrachlorodibenzo-p-dioxin". D. G. Patterson, J.S. Holler, D.F. Grote, L.R. Alexander, C.R. Lapeza, R.C. O'Connor and J.A. Liddle. Environ. Toxicol. Chem. 5, 355-360 (1986).

2. "Method 8290: Analytical Procedures and Quality Assurance for Multimedia Analysis of Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry". Y. Tondeur and W.F. Beckert. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV.

3. "Carcinogens - Working with Carcinogens", Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control. National Institute for Occupational Safety and Health. Publication No. 77-206, August 1977.

4. "OSHA Safety and Health Standards, General Industry", (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206 (revised January 1976).

5. "Safety in Academic Chemistry Laboratories", American Chemical Society Publication, Committee on Chemical Safety (3rd Edition, 1979.)

6. "Hybrid HRGC/MS/MS Method for the Characterization of Tetrachlorinated Dibenzo-p-dioxins in Environmental Samples." Y. Tondeur, W.J. Niederhut, S.R. Missler, and J.E. Campana, Mass Spectrom. 14, 449-456 (1987).

7. USEPA National Dioxin Study - Phase II, "Analytical Procedures and Quality Assurance Plan for the Determination of PCDD/PCDF in Fish", EPA-Duluth, October 26, 1987.

11.0 SAFETY

11.1 The following safety practices are excerpts from EPA Method 613, Sec. 4 (July 1982 version) and amended for use in conjunction with this method. The 2,3,7,8-TCDD isomer has been found to be acnegenic, carcinogenic, and teratogenic in laboratory animal studies. Other PCDDs and PCDFs containing chlorine atoms in positions 2,3,7,8 are known to have toxicities comparable to that of 2,3,7,8-TCDD. The analyst should note that finely divided dry soils contaminated with PCDDs and PCDFs are particularly hazardous because of the potential for inhalation and ingestion. It is recommended that such samples be processed in a confined environment, such as a hood or a glove box. Laboratory personnel handling these types of samples should wear masks fitted with charcoal filters to prevent inhalation of dust.

11.2 The toxicity or carcinogenicity of each reagent used in this method is not precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be kept to a minimum. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should

be made available to all personnel involved in the chemical analysis of samples suspected to contain PCDDs and/or PCDFs. Additional references to laboratory safety are given in references 3, 4 and 5.

11.3 Each laboratory must develop a strict safety program for the handling of PCDDs and PCDFs. The laboratory practices listed below are recommended.

11.3.1 Contamination of the laboratory will be minimized by conducting most of the manipulations in a hood.

11.3.2 The effluents of sample splitters for the gas chromatograph and roughing pumps on the HRGC/HRMS system should pass through either a column of activated charcoal or be bubbled through a trap containing oil or high boiling alcohols.

11.3.3 Liquid waste should be dissolved in methanol or ethanol and irradiated with ultraviolet light at a wavelength less than 290 nm for several days (use F 40 BL lamps, or equivalent). Using this analytical method, analyze the irradiated liquid wastes and dispose of the solutions when 2,3,7,8-TCDD and -TCDF congeners can no longer be detected.

11.4 The following precautions were issued by Dow Chemical U.S.A. (revised 11/78) for safe handling of 2,3,7,8-TCDD in the laboratory and amended for use in conjunction with this method.

11.4.1 The following statements on safe handling are as complete as possible on the basis of available toxicological information. The precautions for safe handling and use are necessarily general in nature since detailed, specific recommendations can be made only for the particular exposure and circumstances of each individual use. Assistance in evaluating the health hazards of particular plant conditions may be obtained from certain consulting laboratories and from State Departments of Health or of Labor, many of which have an industrial health service. The 2,3,7,8-TCDD isomer is extremely toxic to certain kinds of laboratory animals. However, it has been handled for years without injury in analytical and biological laboratories. Many techniques used in handling radioactive and infectious materials are applicable to 2,3,7,8-TCDD.

11.4.1.1 Protective Equipment: Throw away plastic gloves, apron or lab coat, safety glasses and laboratory hood adequate for radioactive work. However, PVC gloves should not be used.

11.4.1.2 Training: Workers must be trained in the proper method of removing contaminated gloves and clothing without contacting the exterior surfaces.

11.4.1.3 Personal Hygiene: Thorough washing of hands and forearms after each manipulation and before breaks (coffee, lunch, and shift).

11.4.1.4 Confinement: Isolated work area, posted with signs, segregated glassware and tools, plastic backed absorbent paper on benchtops.

11.4.1.5 Waste: Good technique includes minimizing contaminated waste. Plastic bag liners should be used in waste cans.

11.4.1.6 Disposal of Hazardous Wastes: Refer to the November 7, 1986 issue of the Federal Register on Land Ban Rulings for details concerning the handling of dioxin containing wastes.

11.4.1.7 Decontamination: Personnel - apply a mild soap with plenty of scrubbing action. Glassware, tools and surfaces - Chlorothene NU Solvent (Trademark of the Dow Chemical Company) is the least toxic solvent shown to be effective. Satisfactory cleaning may be accomplished by rinsing with Chlorothene, then washing with a detergent and water. Dish water may be disposed to the sewer after percolation through a charcoal bed filter. It is prudent to minimize solvent wastes because they require special disposal through commercial services that are expensive.

11.4.1.8 Laundry: Clothing known to be contaminated should be disposed with the precautions described under "Disposal of Hazardous Wastes". Laboratory coats or other clothing worn in 2,3,7,8-TCDD work area may be laundered. Clothing should be collected in plastic bags. Persons who convey the bags and launder the clothing should be advised of the hazard and trained in proper handling. The clothing may be put into a washer without contact if the launderer knows the problem. The washer should be run through one full cycle before being used again for other clothing.

11.4.1.9 Wipe Tests: A useful method for determining cleanliness of work surfaces and tools is to wipe the surface with a piece of filter paper, extract the filter paper and analyze the extract.

NOTE: A procedure for the collection, handling, analysis, and reporting requirements of wipe tests performed within the laboratory is described in Attachment A. The results and decision making processes are based on the presence of 2,3,7,8-substituted PCDDs/PCDFs.

11.4.1.10 Inhalation: Any procedure that may generate airborne contamination must be carried out with good ventilation. Gross losses to a ventilation system must not be allowed. Handling of the dilute solutions normally used in analytical and animal work presents no significant inhalation hazards except in case of an accident.

11.4.1.11 Accidents: Remove contaminated clothing immediately, taking precautions not to contaminate skin or other articles. Wash exposed skin vigorously and repeatedly until medical attention is obtained.

Attachment A

PROCEDURES FOR THE COLLECTION, HANDLING, ANALYSIS, AND REPORTING OF WIPE TESTS PERFORMED WITHIN THE LABORATORY

This procedure is designed for the periodic evaluation of potential contamination by 2,3,7,8-substituted PCDD/PCDF congeners of the working areas inside the laboratory.

A.1 Perform the wipe tests on surface areas of two inches by one foot with glass fiber paper saturated with distilled in glass acetone using a pair of clean stainless steel forceps. Use one wiper for each of the designated areas. Combine the wipers to one composite sample in an extraction jar containing 200 mL distilled in glass acetone. Place an equal number of unused wipers in 200 mL acetone and use this as a control. Add 100 µL of the sample fortification solution to each jar containing used or unused wipers (Sec. 5.8).

A.1.1 Close the jar containing the wipers and the acetone and extract for 20 minutes using a wrist action shaker. Transfer the extract into a KD apparatus fitted with a concentration tube and a three ball Snyder column. Add two Teflon™ or Carborundum ™ boiling chips and concentrate the extract to an apparent volume of 1.0 mL on a steam bath. Rinse the Snyder column and the KD assembly with two 1 mL portions of hexane into the concentrator tube, and concentrate its contents to near dryness with a gentle stream of nitrogen. Add 1.0 mL hexane to the concentrator tube and swirl the solvent on the walls.

A.1.2 Prepare a neutral alumina column as described in Sec. 7.5.2.2 and follow the steps outlined in Secs. 7.5.2.3 through 7.5.2.5.

A.1.3 Add 10 µL of the recovery standard solution as described in Sec. 7.5.3.6.

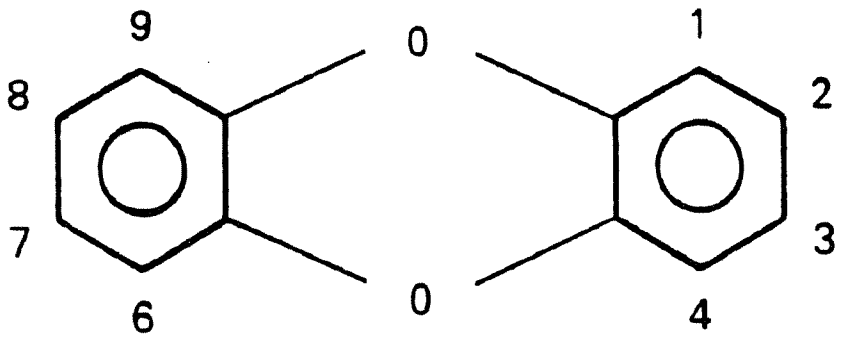
A.2 Concentrate the contents of the vial to a final volume of 10 µL (either in a minivial or in a capillary tube). Inject 2 µL of each extract (wipe and control) onto a capillary column and analyze for 2,3,7,8-substituted PCDDs/PCDFs as specified in the analytical method in Sec. 7.8. Perform calculations according to Sec. 7.9.

A.3 Report the presence of 2,3,7,8-substituted PCDDs and PCDFs as a quantity (pg or ng) per wipe test experiment (WTE). Under the conditions outlined in this analytical protocol, a lower limit of calibration of 10 pg/WTE is expected for 2,3,7,8-TCDD. A positive response for the blank (control) is defined as a signal in the TCDD retention time window at any of the masses monitored which is equivalent to or above 3 pg of 2,3,7,8-TCDD per WTE. For other congeners, use the multiplication factors listed in Table 1, footnote (a) (e.g., for OCDD, the lower MCL is 10 x 5 = 50 pg/WTE and the positive response for the blank would be 3 x 5 = 15 pg). Also, report the recoveries of the internal standards during the simplified cleanup procedure.

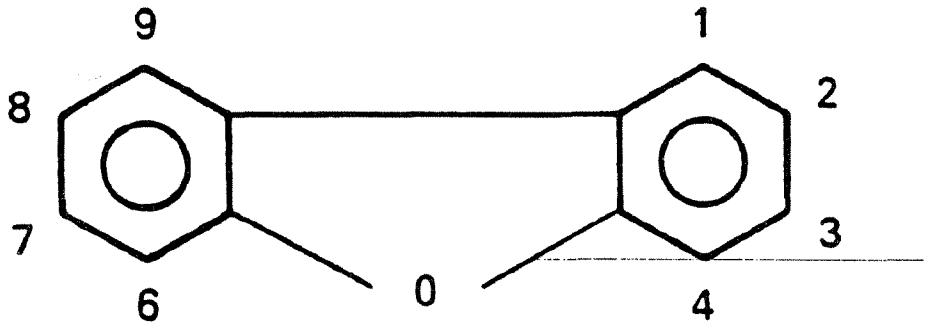
A.4 At a minimum, wipe tests should be performed when there is evidence of contamination in the method blanks.

A.5 An upper limit of 25 pg per TCDD isomer and per wipe test experiment is allowed (use multiplication factors listed in footnote (a) from Table 1 for other congeners). This value corresponds to 2½ times the lower calibration limit of the analytical method. Steps to correct the contamination must be taken whenever these levels are exceeded. To that effect, first vacuum the working places (hoods, benches, sink) using a vacuum cleaner equipped with a high efficiency particulate absorbent (HEPA) filter and then wash with a detergent. A new set of wipes should be analyzed before anyone is allowed to work in the dioxin area of the laboratory after corrective action has been taken.

Figure 1.



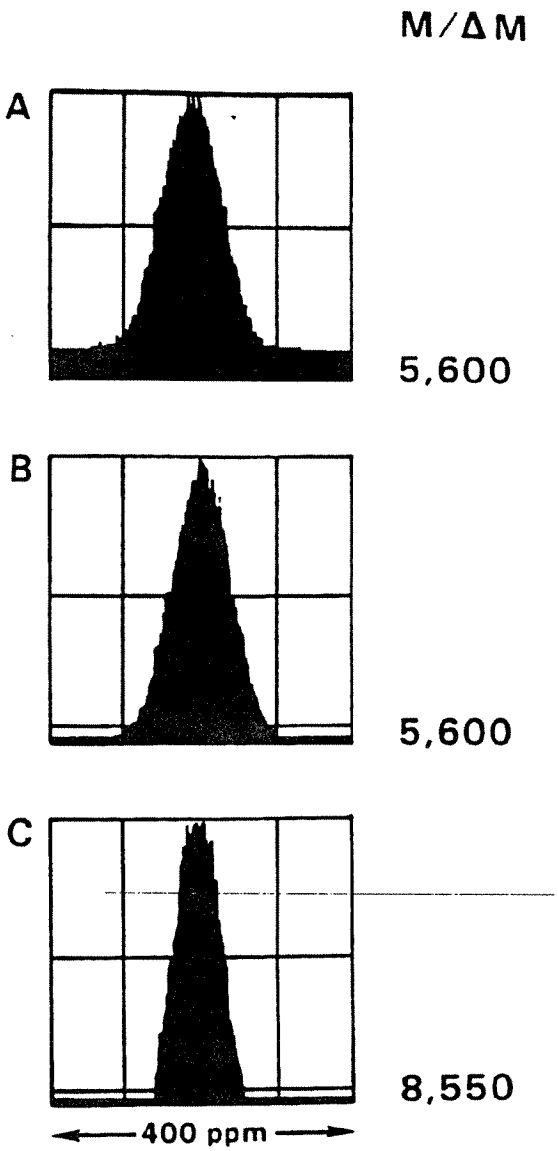
Dibenzodioxin



Dibenzofuran

General structures of dibenzo-p-dioxin and dibenzofuran.

Figure 2.



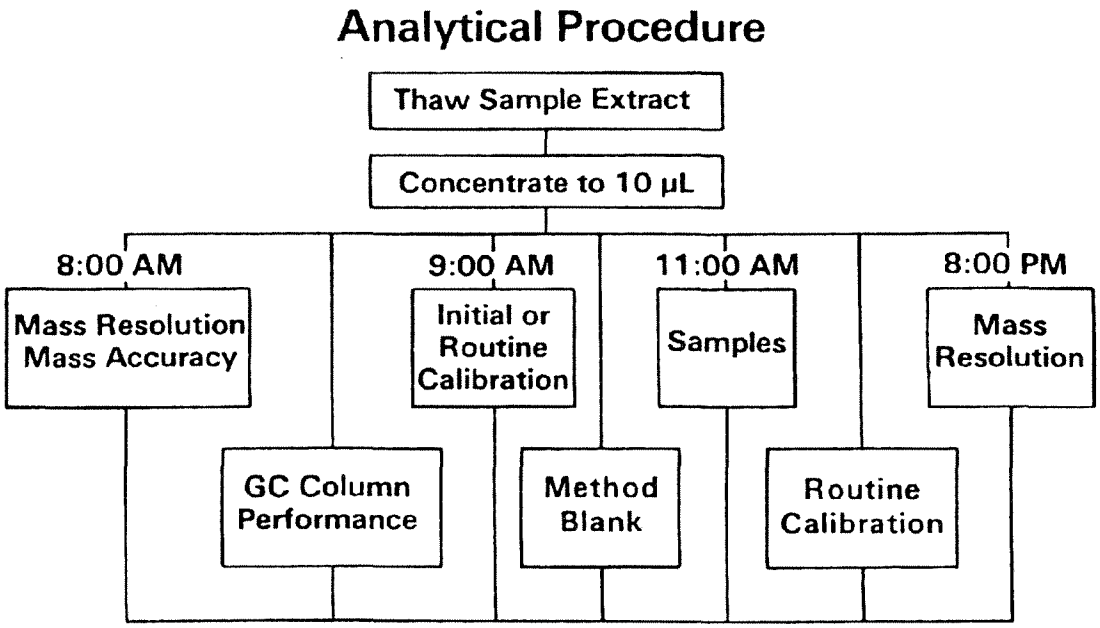
Peak profile displays demonstrating the effect of the detector zero on the measured resolving power. In this example, the true resolving power is 5,600.

A) The zero was set too high; no effect is observed upon the measurement of the resolving power.

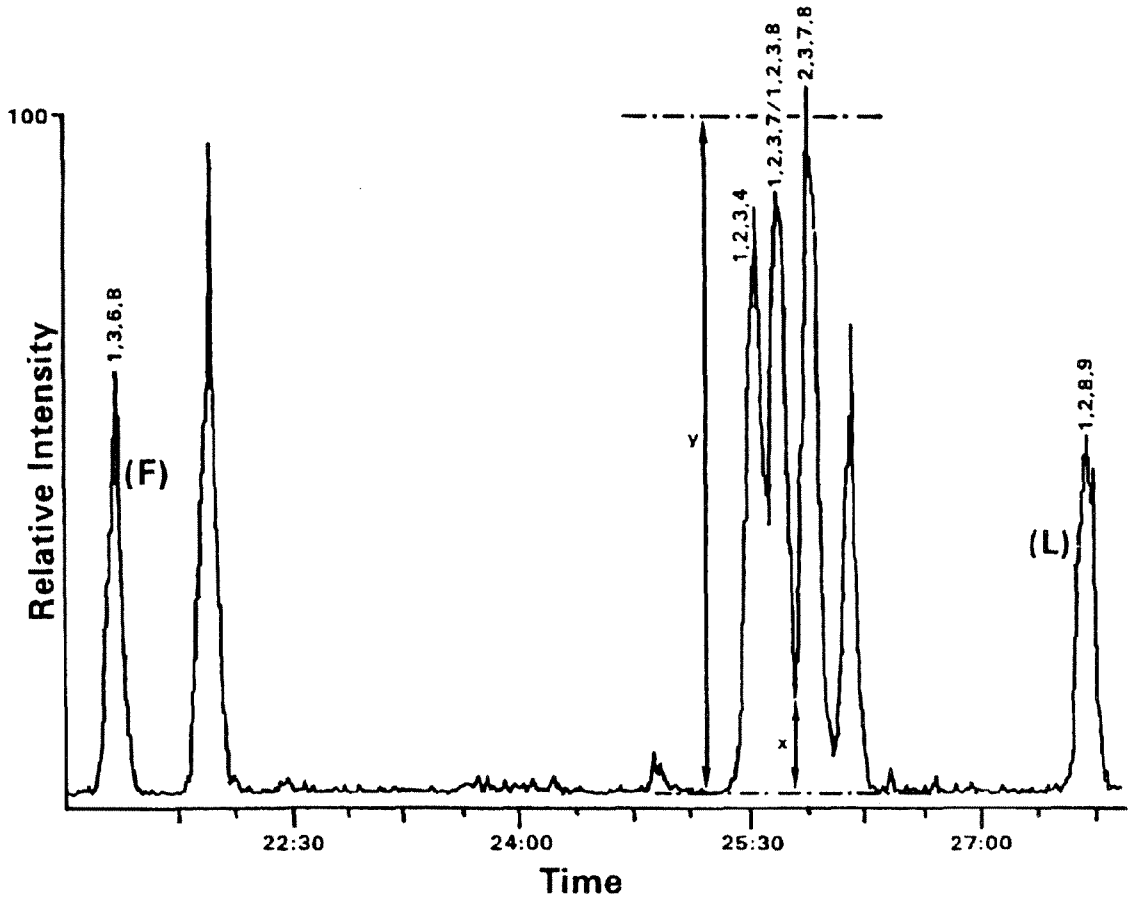
B) The zero was adjusted properly.

C) The zero was set too low; this results in overestimating the actual resolving power because the peak-to-peak noise cannot be measured accurately.

Figure 3.

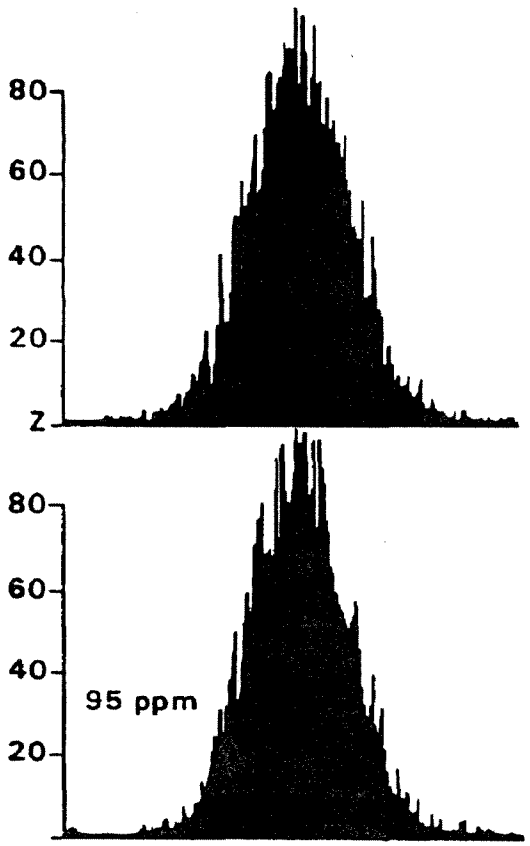


Typical 12 hour analysis sequence of events.
Figure 4.



Selected ion current profile for m/z 322 (TCDDs) produced by MS analysis of the GC performance check solution on a 60 m DB-5 fused silica capillary column under the conditions listed in Sec. 7.6.

Figure 5.



Ref. mass 304.9824 Peak top
Span. 200 ppm

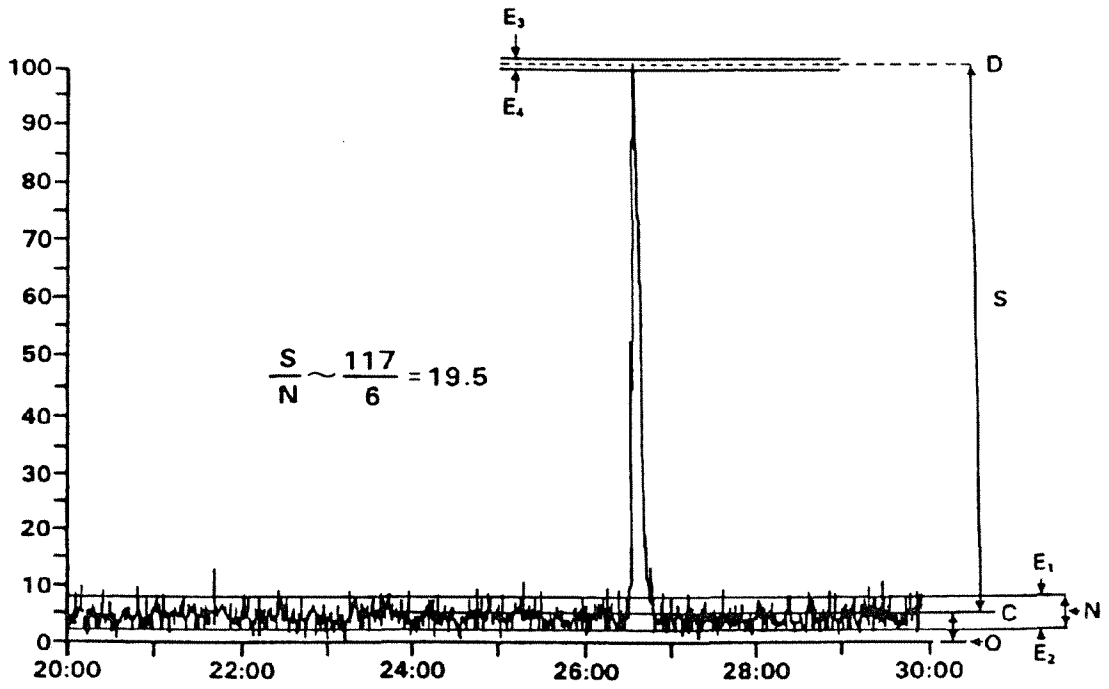
System file name	YVES150
Data file name	A:85Z567
Resolution	10000
Group number	1
Ionization mode	EI+
Switching	VOLTAGE
Ref. masses	304.9824
	380.9260

M/ΔM~10,500

Channel B 380.9260 Lock mass
Span 200 ppm

Peak profiles representing two PFK reference ions at m/z 305 and 381. The resolution of the high-mass signal is 95 ppm at 5 percent of the peak height; this corresponds to a resolving power M/ΔM of 10,500 (10 percent valley definition).

Figure 6.



Manual determination of S/N.

The peak height (S) is measured between the mean noise (lines C and D). These mean signal values are obtained by tracing the line between the baseline average noise extremes, E1 and E2, and between the apex average noise extremes, E3 and E4, at the apex of the signal.

NOTE: It is imperative that the instrument interface amplifier electronic zero offset be set high enough so that negative going baseline noise is recorded.

Table 1.

Types of Matrices, Sample Sizes and 2,3,7,8-TCDD-Based Method Calibration Limits (Parts per Trillion)

	Water	Soil Sediment Paper Pulp[b]	Fly Ash	Fish Tissue[c]	Human Adipose Tissue	Sludges, Fuel Oil	Still-Bottom
Lower MCL[a]	0.01	1.0	1.0	1.0	1.0	5.0	10
Upper MCL[a]	2	200	200	200	200	1000	2000
Weight (g)	1000	10	10	20	10	2	1
IS Spiking Levels (ppt)	1	100	100	100	100	500	1000
Final Extr. Vol. (µL)[d]	10-50	10-50	50	10-50	10-50	50	50

a For other congeners multiply the values by 1 for TCDF/PeCDD/PeCDF, by 2.5 for HxCDD/HxCDF/HpCDD/HpCDF, and by 5 for OCDD/OCDF.

b Sample dewatered according to Sec. 6.5.

c One half of the extract from the 20 g sample is used for determination of lipid content (Sec. 7.2.2).

d See Sec. 7.8.1, Note.

NOTE: Chemical reactor residues are treated as still bottoms if their appearances so suggest.

Table 2.

Composition of the Sample Fortification and Recovery Standard Solutions[a]

Analyte	Sample Fortification Solution Concentration (pg/µL; Solvent: Nonane)	Recovery Standard Solution Concentration (pg/µL; Solvent: Nonane)
$^{13}C_{12}$-2,3,7,8-TCDD	10	--
$^{13}C_{12}$-2,3,7,8-TCDF	10	--
$^{13}C_{12}$-1,2,3,4-TCDD	--	50
$^{13}C_{12}$-1,2,3,7,8-PeCDD	10	--
$^{13}C_{12}$-1,2,3,7,8-PeCDF	10	--
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	25	--
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	25	--
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD	--	50
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	25	--
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF	25	--
$^{13}C_{12}$-OCDD	50	--

(a) These solutions should be made freshly every day because of the possibility of adsorptive losses to glassware. If these solutions are to be kept for more than one day, then the sample fortification solution concentrations should be increased ten fold, and the recovery standard solution concentrations should be doubled. Corresponding adjustments of the spiking volumes must then be made.

Table 3.

The Fifteen 2,3,7,8-Substituted PCDD and PCDF Congeners

PCDD	PCDF
2,3,7,8-TCDD(*)	2,3,7,8-TCDF(*)
1,2,3,7,8-PeCDD(*)	1,2,3,7,8-PeCDF(*)
1,2,3,6,7,8-HxCDD(*)	2,3,4,7,8-PeCDF
1,2,3,4,7,8-HxCDD	1,2,3,6,7,8-HxCDF
1,2,3,7,8,9-HxCDD(+)	1,2,3,7,8,9-HxCDF
1,2,3,4,6,7,8-HpCDD(*)	1,2,3,4,7,8-HxCDF(*)
	2,3,4,6,7,8-HxCDF
	1,2,3,4,6,7,8-HpCDF(*)
	1,2,3,4,7,8,9-HpCDF

(*) The 13C-labeled analogue is used as an internal standard.

(+) The 13C-labeled analogue is used as a recovery standard.

Table 4.

Isomers of Chlorinated Dioxins and Furans as a Function of the Number of Chlorine Atoms

Number of Chlorine Atoms	Number of Dioxin Isomers	Number of 2,3,7,8 Isomers	Number of Furan Isomers	Number of 2,3,7,8 Isomers
1	2	---	4	---
2	10	---	16	---
3	14	---	28	---
4	22	1	38	1
5	14	1	28	2
6	10	3	16	4
7	2	1	4	2
8	1	1	1	1
Total	75	7	135	10

Table 5.

High-Resolution Concentration Calibration Solutions

Compound	HRCC	Concentration (pg/µL, in Nonane) 5	4	3	2	1
Unlabeled Analytes						
2,3,7,8-TCDD		200	50	10	2.5	1
2,3,7,8-TCDF		200	50	10	2.5	1
1,2,3,7,8-PeCDD		500	125	25	6.25	2.5
1,2,3,7,8-PeCDF		500	125	25	6.25	2.5
2,3,4,7,8-PeCDF		500	125	25	6.25	2.5
1,2,3,4,7,8-HxCDD		500	125	25	6.25	2.5
1,2,3,6,7,8-HxCDD		500	125	25	6.25	2.5
1,2,3,7,8,9-HxCDD		500	125	25	6.25	2.5
1,2,3,4,7,8-HxCDF		500	125	25	6.25	2.5
1,2,3,6,7,8-HxCDF		500	125	25	6.25	2.5
1,2,3,7,8,9-HxCDF		500	125	25	6.25	2.5
2,3,4,6,7,8-HxCDF		500	125	25	6.25	2.5
1,2,3,4,6,7,8-HpCDD		500	125	25	6.25	2.5
1,2,3,4,6,7,8-HpCDF		500	125	25	6.25	2.5
1,2,3,4,7,8,9-HpCDF		500	125	25	6.25	2.5
OCDD		1,000	250	50	12.5	5
OCDF		1,000	250	50	12.5	5
Internal Standards						
$^{13}C_{12}$-2,3,7,8-TCDD		50	50	50	50	50
$^{13}C_{12}$-2,3,7,8-TCDF		50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDD		50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8-PeCDF		50	50	50	50	50
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD		125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF		125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD		125	125	125	125	125
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF		125	125	125	125	125
$^{13}C_{12}$-OCDD		250	250	250	250	250
Recovery Standards						
$^{13}C_{12}$-1,2,3,4-TCDD[a]		50	50	50	50	50
$^{13}C_{12}$-1,2,3,7,8,9-HxCDD[b]		125	125	125	125	125

[a] Used for recovery determinations of TCDD, TCDF, PeCDD and PeCDF internal standards.

[b] Used for recovery determinations of HxCDD, HxCDF, HpCDD, HpCDF and OCDD internal standards.

Table 6.

Ions Monitored for HRGC/HRMS Analysis of PCDDs/PCDFs

Descriptor	Accurate[a] Mass	Ion ID	Elemental Composition	Analyte
1	303.9016	M	$C_{12}H_4{}^{35}Cl_4O$	TCDF
	305.8987	M+2	$C_{12}H_4{}^{35}Cl_3{}^{37}ClO$	TCDF
	315.9419	M	$^{13}C_{12}H_4{}^{35}Cl_4O$	TCDF (S)
	317.9389	M+2	$^{13}C_{12}H_4{}^{35}Cl_3{}^{37}ClO$	TCDF (S)
	319.8965	M	$C_{12}H_4{}^{35}Cl_4O_2$	TCDD
	321.8936	M+2	$C_{12}H_4{}^{35}Cl_3{}^{37}ClO_2$	TCDD
	331.9368	M	$^{13}C_{12}H_4{}^{35}Cl_4O_2$	TCDD (S)
	333.9338	M+2	$^{13}C_{12}H_4{}^{35}Cl_3{}^{37}ClO_2$	TCDD (S)
	375.8364	M+2	$C_{12}H_4{}^{35}Cl_5{}^{37}ClO$	HxCDPE
	[354.9792]	LOCK	C_9F_{13}	PFK
2	339.8597	M+2	$C_{12}H_3{}^{35}Cl_4{}^{37}ClO$	PeCDF
	341.8567	M+4	$C_{12}H_3{}^{35}Cl_3{}^{37}Cl_2O$	PeCDF
	351.9000	M+2	$^{13}C_{12}H_3{}^{35}Cl_4{}^{37}ClO$	PeCDF (S)
	353.8970	M+4	$^{13}C_{12}H_3{}^{35}Cl_3{}^{37}Cl_2O$	PeCDF (S)
	355.8546	M+2	$C_{12}H_3{}^{35}Cl_4{}^{37}ClO_2$	PeCDD
	357.8516	M+4	$C_{12}H_3{}^{35}Cl_3{}^{37}Cl_2O_2$	PeCDD
	367.8949	M+2	$^{13}C_{12}H_3{}^{35}Cl_4{}^{37}ClO_2$	PeCDD (S)
	369.8919	M+4	$^{13}C_{12}H_3{}^{35}Cl_3{}^{37}Cl_2O_2$	PeCDD (S)
	409.7974	M+2	$C_{12}H_3{}^{35}Cl_6{}^{37}ClO$	HpCDPE
	[354.9792]	LOCK	C_9F_{13}	PFK
3	373.8208	M+2	$C_{12}H_2{}^{35}Cl_5{}^{37}ClO$	HxCDF
	375.8178	M+4	$C_{12}H_2{}^{35}Cl_4{}^{37}Cl_2O$	HxCDF
	383.8639	M	$^{13}C_{12}H_2{}^{35}Cl_6O$	HxCDF (S)
	385.8610	M+2	$^{13}C_{12}H_2{}^{35}Cl_5{}^{37}ClO$	HxCDF (S)
	389.8156	M+2	$C_{12}H_2{}^{35}Cl_5{}^{37}ClO_2$	HxCDD
	391.8127	M+4	$C_{12}H_2{}^{35}Cl_4{}^{37}Cl_2O_2$	HxCDD
	401.8559	M+2	$^{13}C_{12}H_2{}^{35}Cl_5{}^{37}ClO_2$	HxCDD (S)
	403.8529	M+4	$^{13}C_{12}H_2{}^{35}Cl_4{}^{37}Cl_2O_2$	HxCDD (S)
	445.7555	M+4	$C_{12}H_2{}^{35}Cl_6{}^{37}Cl_2O$	OCDPE
	[430.9728]	LOCK	C_9F_{17}	PFK

Table 6.
Continued

Descriptor	Accurate[a] Mass	Ion ID	Elemental Composition	Analyte
4	407.7818	M+2	$C_{12}H^{35}Cl_6{}^{37}ClO$	HpCDF
	409.7788	M+4	$C_{12}H^{35}Cl_5{}^{37}Cl_2O$	HpCDF
	417.8250	M	$^{13}C_{12}H^{35}Cl_7O$	HpCDF (S)
	419.8220	M+2	$^{13}C_{12}H^{35}Cl_6{}^{37}ClO$	HpCDF
	423.7767	M+2	$C_{12}H^{35}Cl_6{}^{37}ClO_2$	HpCDD
	425.7737	M+4	$C_{12}H^{35}Cl_5{}^{37}Cl_2O_2$	HpCDD
	435.8169	M+2	$^{13}C_{12}H^{35}Cl_6{}^{37}ClO_2$	HpCDD (S)
	437.8140	M+4	$^{13}C_{12}H^{35}Cl_5{}^{37}Cl_2O_2$	HpCDD (S)
	479.7165	M+4	$C_{12}H^{35}Cl_7{}^{37}Cl_2O$	NCDPE
	[430.9728]	LOCK	C_9F_{17}	PFK
5	441.7428	M+2	$C_{12}{}^{35}Cl_7{}^{37}ClO$	OCDF
	443.7399	M+4	$C_{12}{}^{35}Cl_6{}^{37}Cl_2O$	OCDF
	457.7377	M+2	$C_{12}{}^{35}Cl_7{}^{37}ClO_2$	OCDD
	459.7348	M+4	$C_{12}{}^{35}Cl_6{}^{37}Cl_2O_2$	OCDD
	469.7780	M+2	$^{13}C_{12}{}^{35}Cl_7{}^{37}ClO_2$	OCDD (S)
	471.7750	M+4	$^{13}C_{12}{}^{35}Cl_6{}^{37}Cl_2O_2$	OCDD (S)
	513.6775	M+4	$C_{12}{}^{35}Cl_8{}^{37}Cl_2O$	DCDPE
	[442.9728]	LOCK	$C_{10}F_{17}$	PFK

[a] The following nuclidic masses were used:

H	=	1.007825	O	=	15.994915
C	=	12.000000	^{35}Cl	=	34.968853
^{13}C	=	13.003355	^{37}Cl	=	36.965903
F	=	18.9984			

S = internal/recovery standard

Table 7.

PCDD and PCDF Congeners Present in the GC Performance Evaluation Solution and Used for Defining the Homologous GC Retention Time Windows on a 60 m DB-5 Column

No. of Chlorine Atoms	PCDD Positional Isomer		PCDF Positional Isomer	
	First Eluter	Last Eluter	First Eluter	Last Eluter
4[a]	1,3,6,8	1,2,8,9	1,3,6,8	1,2,8,9
5	1,2,4,6,8/ 1,2,4,7,9	1,2,3,8,9	1,3,4,6,8	1,2,3,8,9
6	1,2,4,6,7,9/ 1,2,4,6,8,9	1,2,3,4,6,7	1,2,3,4,6,8	1,2,3,4,8,9
7	1,2,3,4,6,7,9	1,2,3,4,6,7,8	1,2,3,4,6,7,8	1,2,3,4,7,8,9
8		1,2,3,4,6,7,8,9		1,2,3,4,6,7,8,9

[a] In addition to these two TCDD isomers, the 1,2,3,4-, 1,2,3,7-, 1,2,3,8-, 2,3,7,8-, $^{13}C_{12}$-2,3,7,8-, and 1,2,3,9-TCDD isomers must also be present as a check of column resolution.

Table 8.

Theoretical Ion Abundance Ratios and Their Control Limits
for PCDDs and PCDFs

Number of Chlorine Atoms	Ion Type	Theoretical Ratio	Control Limits lower	Control Limits upper
4	M/M+2	0.77	0.65	0.89
5	M+2/M+4	1.55	1.32	1.78
6	M+2/M+4	1.24	1.05	1.43
6[a]	M/M+2	0.51	0.43	0.59
7[b]	M/M+2	0.44	0.37	0.51
7	M+2/M+4	1.04	0.88	1.20
8	M+2/M+4	0.89	0.76	1.02

[a] Used only for ^{13}C-HxCDF (IS).

[b] Used only for ^{13}C-HpCDF (IS).

Table 9.

Relative Response Factor [RF (number)] Attributions

Number	Specific Congener Name
1	2,3,7,8-TCDD (and total TCDDs)
2	2,3,7,8-TCDF (and total TCDFs)
3	1,2,3,7,8-PeCDD (and total PeCDDs)
4	1,2,3,7,8-PeCDF
5	2,3,4,7,8-PeCDF
6	1,2,3,4,7,8-HxCDD
7	1,2,3,6,7,8-HxCDD
8	1,2,3,7,8,9-HxCDD
9	1,2,3,4,7,8-HxCDF
10	1,2,3,6,7,8-HxCDF
11	1,2,3,7,8,9-HxCDF
12	2,3,4,6,7,8-HxCDF
13	1,2,3,4,6,7,8-HpCDD (and total HpCDDs)
14	1,2,3,4,6,7,8-HpCDF
15	1,2,3,4,7,8,9-HpCDF
16	OCDD
17	OCDF
18	$^{13}C_{12}$-2,3,7,8-TCDD
19	$^{13}C_{12}$-2,3,7,8-TCDF
20	$^{13}C_{12}$-1,2,3,7,8-PeCDD
21	$^{13}C_{12}$-1,2,3,7,8-PeCDF
22	$^{13}C_{12}$-1,2,3,6,7,8-HxCDD
23	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF
24	$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD
25	$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF
26	$^{13}C_{12}$-OCDD
27	Total PeCDFs
28	Total HxCDFs
29	Total HxCDDs
30	Total HpCDFs

Table 10.

2,3,7,8-TCDD Toxicity Equivalency Factors (TEFs) for the Polychlorinated Dibenzodioxins and Dibenzofurans

Number	Compound(s)	TEF[a]
1	2,3,7,8-TCDD	1.00
2	1,2,3,7,8-PeCDD	0.50
3	1,2,3,6,7,8-HxCDD	0.10
4	1,2,3,7,8,9-HxCDD	0.10
5	1,2,3,4,7,8-HxCDD	0.10
6	1,2,3,4,6,7,8-HpCDD	0.01
7	1,2,3,4,6,7,8,9-OCDD	0.001
8	2,3,7,8-TCDF	0.1
9	1,2,3,7,8-PeCDF	0.05
10	2,3,4,7,8-PeCDF	0.5
11	1,2,3,6,7,8-HxCDF	0.1
12	1,2,3,7,8,9-HxCDF	0.1
13	1,2,3,4,7,8-HxCDF	0.1
14	2,3,4,6,7,8-HxCDF	0.1
15	1,2,3,4,6,7,8-HpCDF	0.01
16	1,2,3,4,7,8,9-HpCDF	0.01
17	1,2,3,4,6,7,8,9-OCDF	0.001

[a] Taken from "Interim Procedures for Estimating Risks Associated with Exposures to Mixtures of Chlorinated Dibenzo-p-Dioxin and -Dibenzofurans (CDDs and CDFs) and 1989 Update", (EPA/625/3-89/016, March 1989).

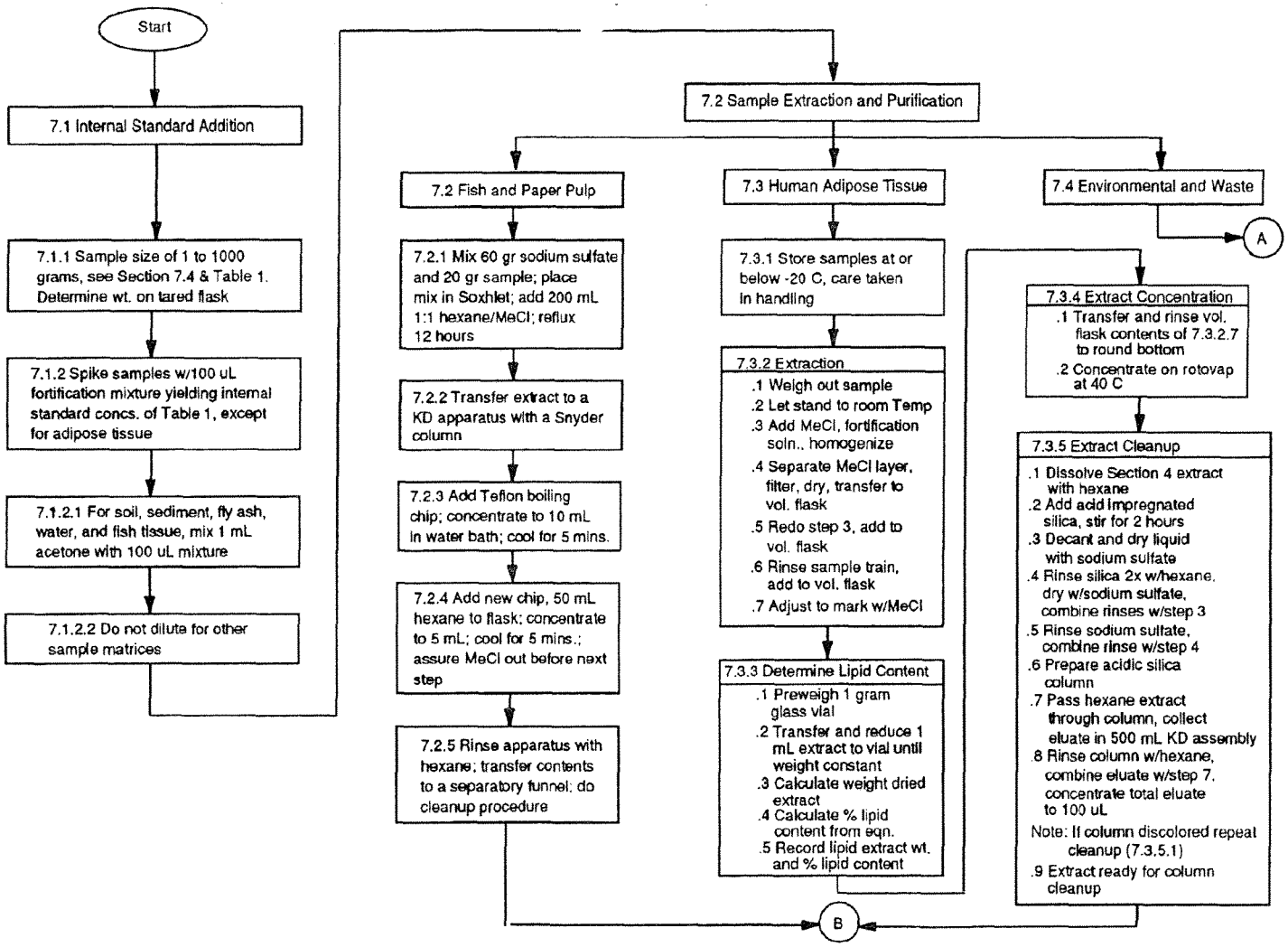
Table 11.

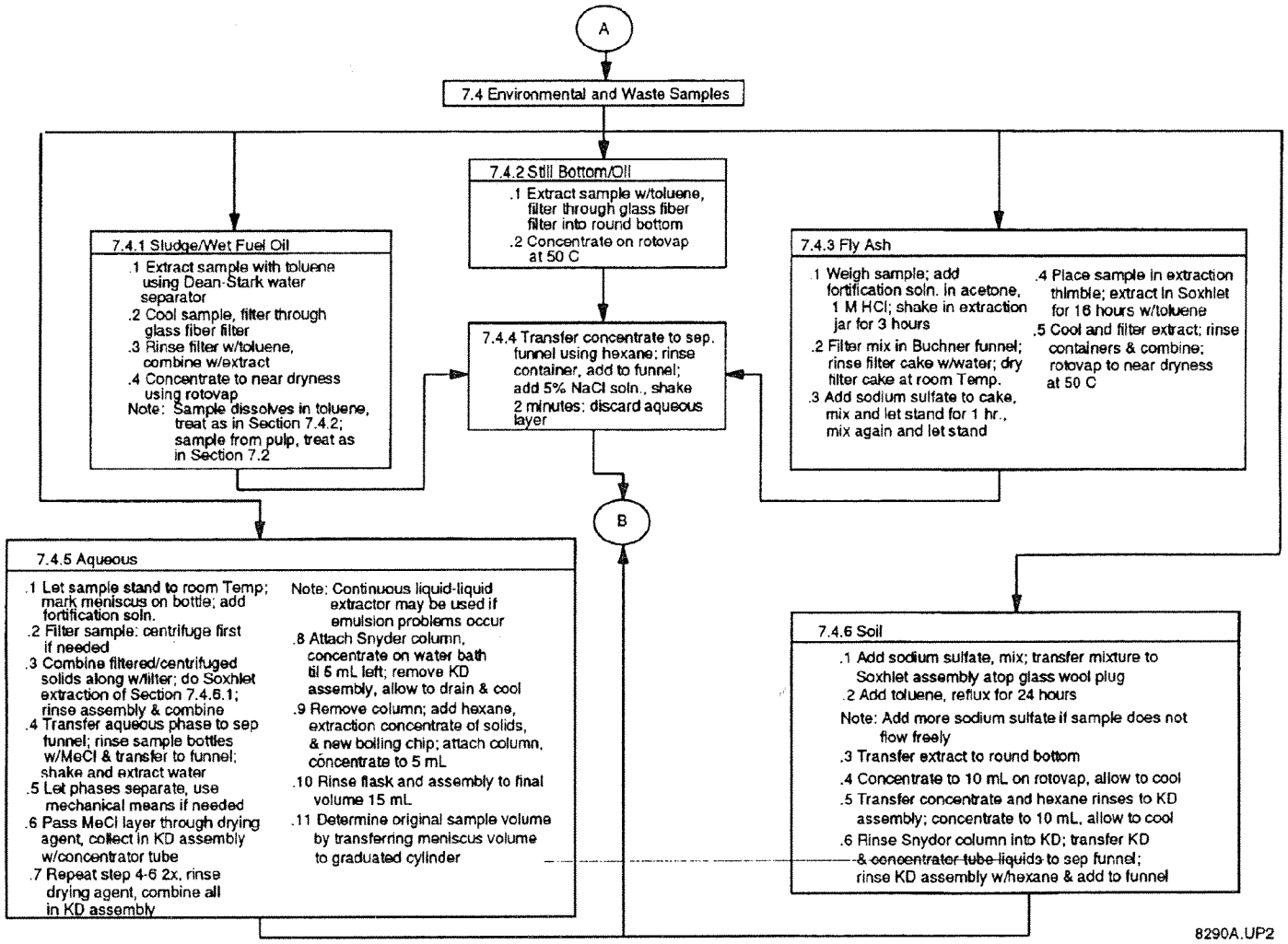
Analyte Relative Retention Time Reference Attributions

Analyte	Analyte RRT Reference[a]
1,2,3,4,7,8-HxCDD	$^{13}C_{12}$-1,2,3,6,7,8-HxCDD
1,2,3,6,7,8-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF
1,2,3,7,8,9-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF
2,3,4,6,7,8-HxCDF	$^{13}C_{12}$-1,2,3,4,7,8-HxCDF

[a] The retention time of 2,3,4,7,8-PeCDF on the DB-5 column is measured relative to $^{13}C_{12}$-1,2,3,7,8-PeCDF and the retention time of 1,2,3,4,7,8,9-HpCDF relative to $^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF.

METHOD 8290
POLYCHLORINATED DIBENZODIOXINS (PCDDs) AND POLYCHLORINATED DIBENZOFURANS (PCDFs)
BY HIGH-RESOLUTION GAS CHROMATOGRAPHY/HIGH-RESOLUTION MASS SPECTROMETRY
(HRGC/HRMS)




A
7.4 Environmental and Waste Samples
7.4.1 Sludge/Wet Fuel Oil
.1 Extract sample with toluene using Dean-Stark water separator
.2 Cool sample, filter through glass fiber filter
.3 Rinse filter w/toluene, combine w/extract
.4 Concentrate to near dryness using rotovap
Note: Sample dissolves in toluene, treat as in Section 7.4.2; sample from pulp, treat as in Section 7.2
7.4.2 Still Bottom/Oil
.1 Extract sample w/toluene, filter through glass fiber filter into round bottom
.2 Concentrate on rotovap at 50 C
7.4.3 Fly Ash
.1 Weigh sample; add fortification soln. in acetone, 1 M HCl; shake in extraction jar for 3 hours
.2 Filter mix in Buchner funnel; rinse filter cake w/water; dry filter cake at room Temp.
.3 Add sodium sulfate to cake, mix and let stand for 1 hr., mix again and let stand
.4 Place sample in extraction thimble; extract in Soxhlet for 16 hours w/toluene
.5 Cool and filter extract; rinse containers & combine; rotovap to near dryness at 50 C
7.4.4 Transfer concentrate to sep. funnel using hexane; rinse container, add to funnel; add 5% NaCl soln., shake 2 minutes; discard aqueous layer
B
7.4.5 Aqueous
.1 Let sample stand to room Temp; mark meniscus on bottle; add fortification soln.
.2 Filter sample: centrifuge first if needed
.3 Combine filtered/centrifuged solids along w/filter; do Soxhlet extraction of Section 7.4.6.1; rinse assembly & combine
.4 Transfer aqueous phase to sep funnel; rinse sample bottles w/MeCl & transfer to funnel; shake and extract water
.5 Let phases separate, use mechanical means if needed
.6 Pass MeCl layer through drying agent, collect in KD assembly w/concentrator tube
.7 Repeat step 4-6 2x, rinse drying agent, combine all in KD assembly
Note: Continuous liquid-liquid extractor may be used if emulsion problems occur
.8 Attach Snyder column, concentrate on water bath til 5 mL left; remove KD assembly, allow to drain & cool
.9 Remove column; add hexane, extraction concentrate of solids, & new boiling chip; attach column, concentrate to 5 mL
.10 Rinse flask and assembly to final volume 15 mL
.11 Determine original sample volume by transferring meniscus volume to graduated cylinder
7.4.6 Soil
.1 Add sodium sulfate, mix; transfer mixture to Soxhlet assembly atop glass wool plug
.2 Add toluene, reflux for 24 hours
Note: Add more sodium sulfate if sample does not flow freely
.3 Transfer extract to round bottom
.4 Concentrate to 10 mL on rotovap, allow to cool
.5 Transfer concentrate and hexane rinses to KD assembly; concentrate to 10 mL, allow to cool
.6 Rinse Snydor column into KD; transfer KD & concentrator tube liquids to sep funnel; rinse KD assembly w/hexane & add to funnel
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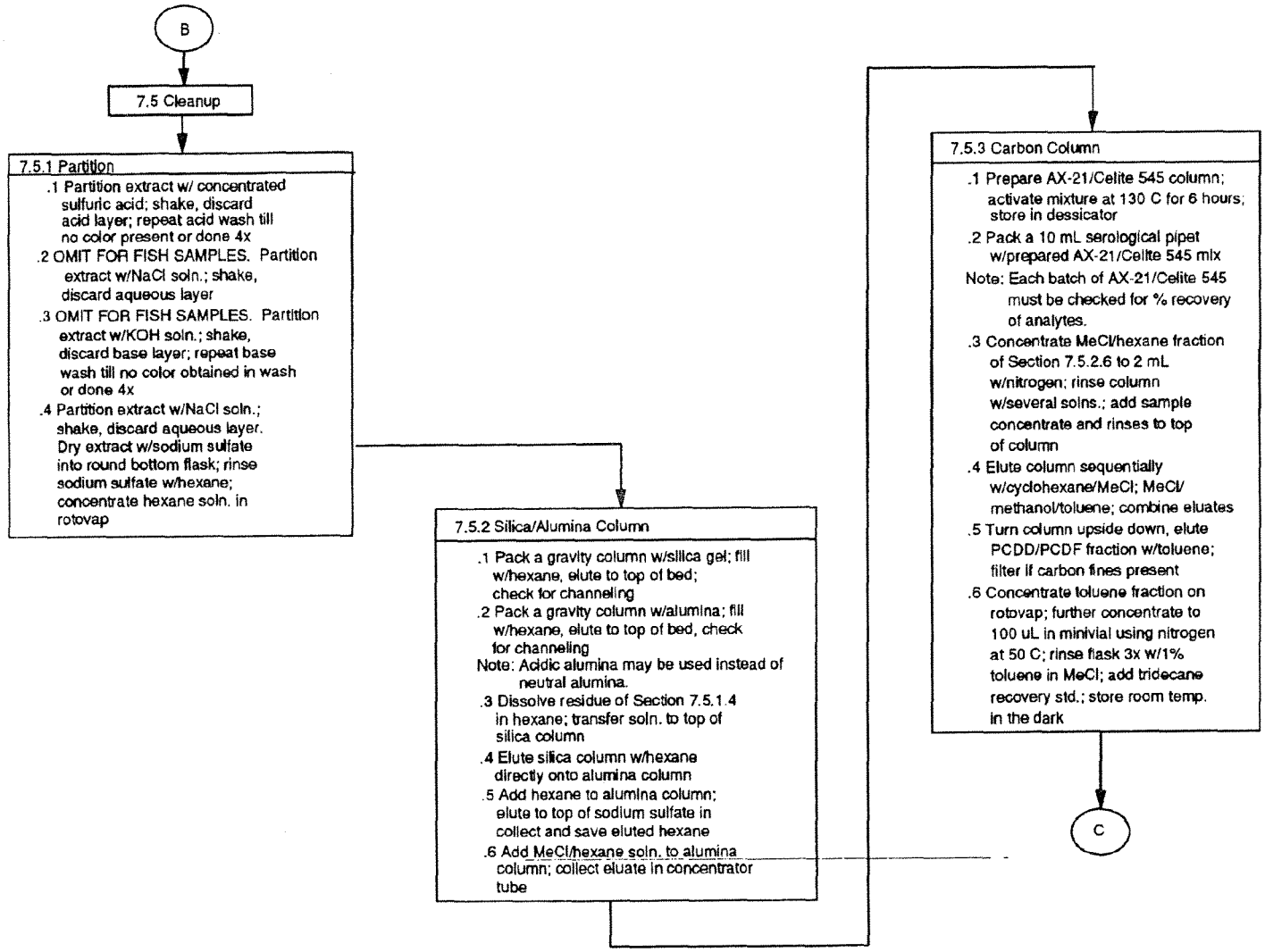
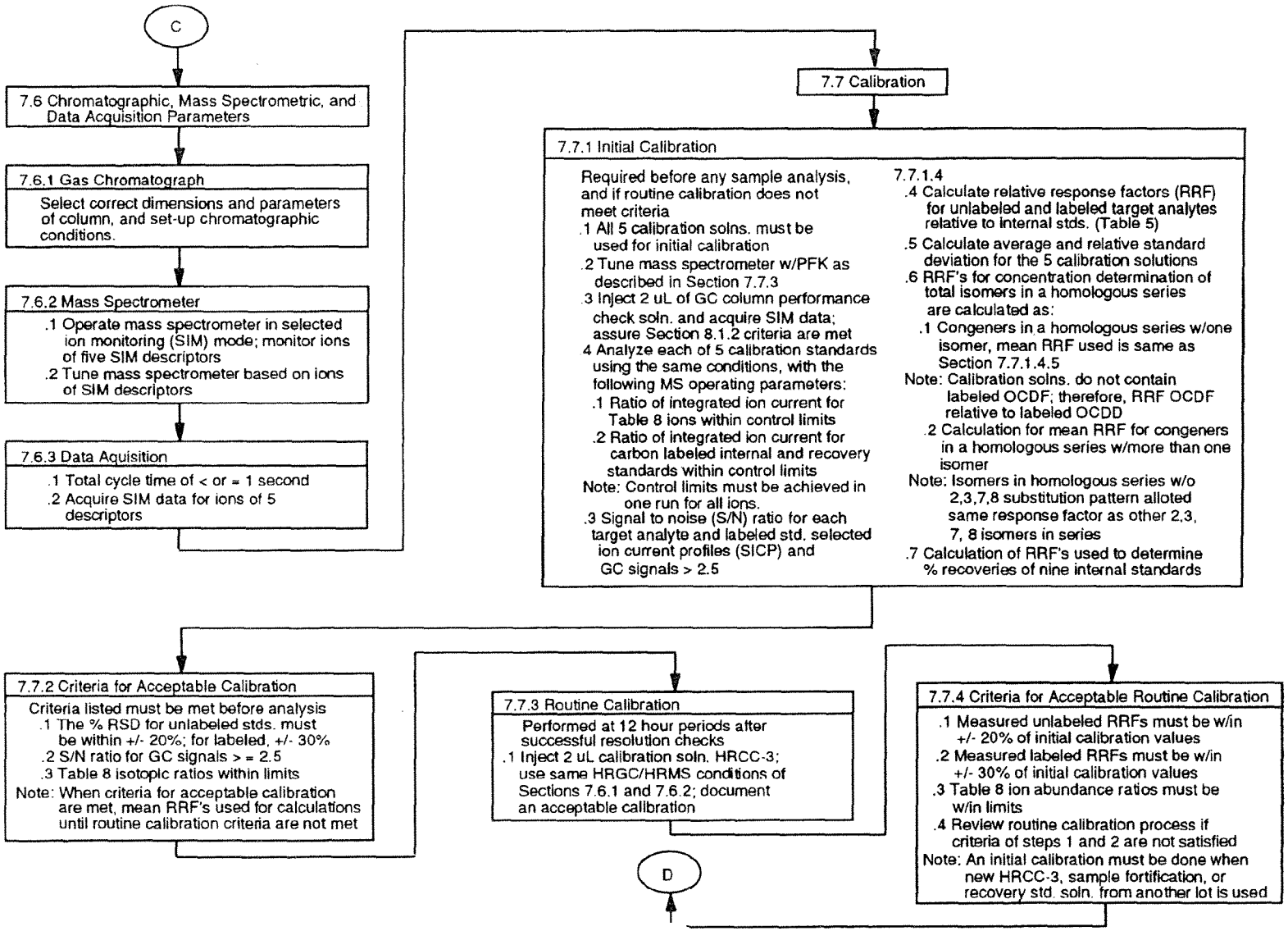

B
7.5 Cleanup
7.5.1 Partition
.1 Partition extract w/ concentrated sulfuric acid; shake, discard acid layer; repeat acid wash till no color present or done 4x
.2 OMIT FOR FISH SAMPLES. Partition extract w/NaCl soln.; shake, discard aqueous layer
.3 OMIT FOR FISH SAMPLES. Partition extract w/KOH soln.; shake, discard base layer; repeat base wash till no color obtained in wash or done 4x
.4 Partition extract w/NaCl soln.; shake, discard aqueous layer. Dry extract w/sodium sulfate into round bottom flask; rinse sodium sulfate w/hexane; concentrate hexane soln. in rotovap
7.5.2 Silica/Alumina Column
.1 Pack a gravity column w/silica gel; fill w/hexane, elute to top of bed; check for channeling
.2 Pack a gravity column w/alumina; fill w/hexane, elute to top of bed, check for channeling
Note: Acidic alumina may be used instead of neutral alumina.
.3 Dissolve residue of Section 7.5.1.4 in hexane; transfer soln. to top of silica column
.4 Elute silica column w/hexane directly onto alumina column
.5 Add hexane to alumina column; elute to top of sodium sulfate in collect and save eluted hexane
.6 Add MeCl/hexane soln. to alumina column; collect eluate in concentrator tube
7.5.3 Carbon Column
.1 Prepare AX-21/Celite 545 column; activate mixture at 130 C for 6 hours; store in dessicator
.2 Pack a 10 mL serological pipet w/prepared AX-21/Celite 545 mix
Note: Each batch of AX-21/Celite 545 must be checked for % recovery of analytes.
.3 Concentrate MeCl/hexane fraction of Section 7.5.2.6 to 2 mL w/nitrogen; rinse column w/several solns.; add sample concentrate and rinses to top of column
.4 Elute column sequentially w/cyclohexane/MeCl; MeCl/methanol/toluene; combine eluates
.5 Turn column upside down, elute PCDD/PCDF fraction w/toluene; filter if carbon fines present
.6 Concentrate toluene fraction on rotovap; further concentrate to 100 uL in minivial using nitrogen at 50 C; rinse flask 3x w/1% toluene in MeCl; add tridecane recovery std.; store room temp. in the dark
C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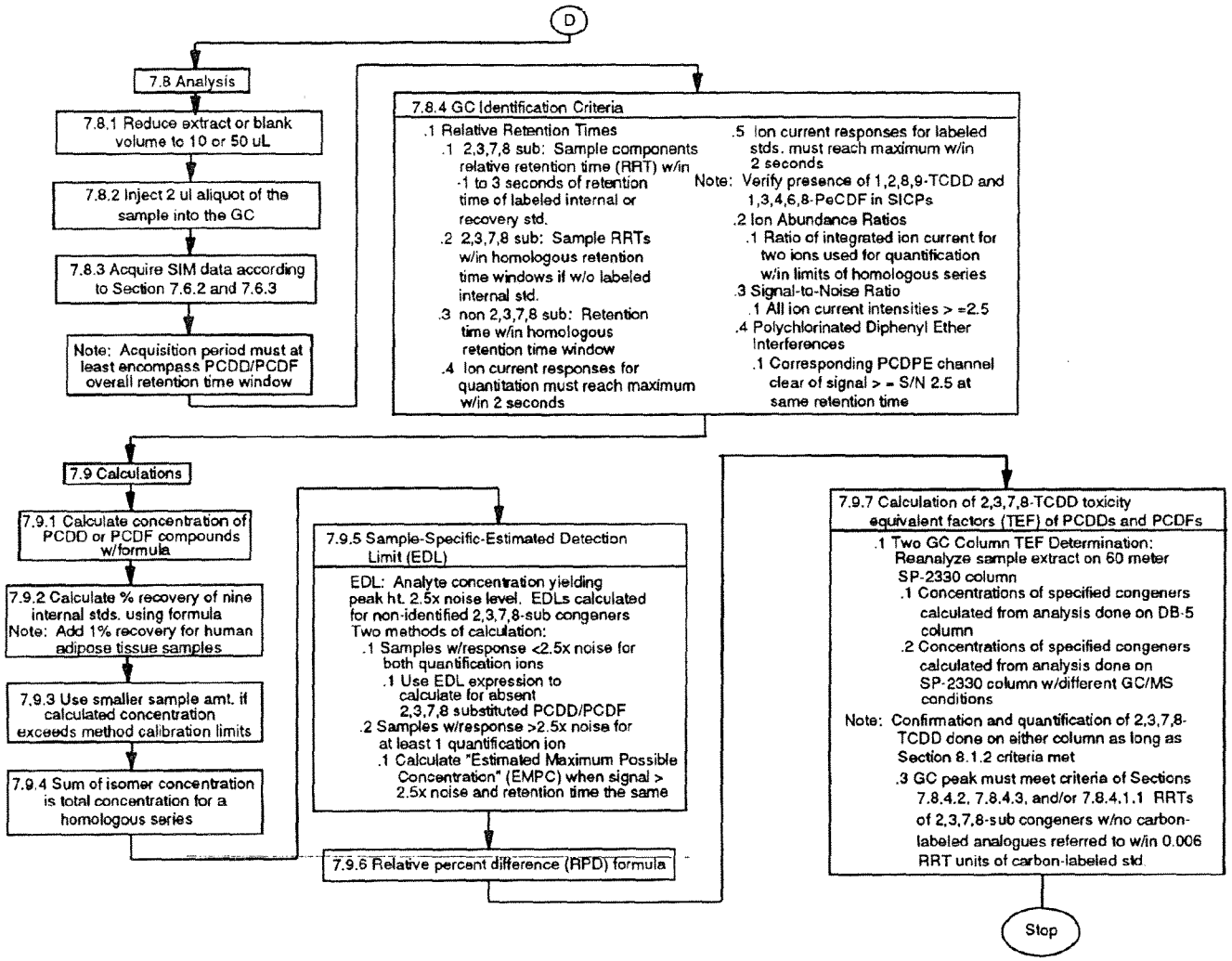
7.6 Chromatographic, Mass Spectrometric, and Data Acquisition Parameters
7.6.1 Gas Chromatograph
Select correct dimensions and parameters of column, and set-up chromatographic conditions.
7.6.2 Mass Spectrometer
.1 Operate mass spectrometer in selected ion monitoring (SIM) mode; monitor ions of five SIM descriptors
.2 Tune mass spectrometer based on ions of SIM descriptors
7.6.3 Data Aquisition
.1 Total cycle time of < or = 1 second
.2 Acquire SIM data for ions of 5 descriptors
7.7 Calibration
7.7.1 Initial Calibration
Required before any sample analysis, and if routine calibration does not meet criteria
.1 All 5 calibration solns. must be used for initial calibration
.2 Tune mass spectrometer w/PFK as described in Section 7.7.3
.3 Inject 2 uL of GC column performance check soln. and acquire SIM data; assure Section 8.1.2 criteria are met
4 Analyze each of 5 calibration standards using the same conditions, with the following MS operating parameters:
.1 Ratio of integrated ion current for Table 8 ions within control limits
.2 Ratio of integrated ion current for carbon labeled internal and recovery standards within control limits
Note: Control limits must be achieved in one run for all ions.
.3 Signal to noise (S/N) ratio for each target analyte and labeled std. selected ion current profiles (SICP) and GC signals > 2.5
7.7.1.4
.4 Calculate relative response factors (RRF) for unlabeled and labeled target analytes relative to internal stds. (Table 5)
.5 Calculate average and relative standard deviation for the 5 calibration solutions
.6 RRF's for concentration determination of total isomers in a homologous series are calculated as:
.1 Congeners in a homologous series w/one isomer, mean RRF used is same as Section 7.7.1.4.5
Note: Calibration solns. do not contain labeled OCDF; therefore, RRF OCDF relative to labeled OCDD
.2 Calculation for mean RRF for congeners in a homologous series w/more than one isomer
Note: Isomers in homologous series w/o 2,3,7,8 substitution pattern alloted same response factor as other 2,3, 7, 8 isomers in series
.7 Calculation of RRF's used to determine % recoveries of nine internal standards
7.7.2 Criteria for Acceptable Calibration
Criteria listed must be met before analysis
.1 The % RSD for unlabeled stds. must be within +/- 20%; for labeled, +/- 30%
.2 S/N ratio for GC signals > = 2.5
.3 Table 8 isotopic ratios within limits
Note: When criteria for acceptable calibration are met, mean RRF's used for calculations until routine calibration criteria are not met
7.7.3 Routine Calibration
Performed at 12 hour periods after successful resolution checks
.1 Inject 2 uL calibration soln. HRCC-3; use same HRGC/HRMS conditions of Sections 7.6.1 and 7.6.2; document an acceptable calibration
7.7.4 Criteria for Acceptable Routine Calibration
.1 Measured unlabeled RRFs must be w/in +/- 20% of initial calibration values
.2 Measured labeled RRFs must be w/in +/- 30% of initial calibration values
.3 Table 8 ion abundance ratios must be w/in limits
.4 Review routine calibration process if criteria of steps 1 and 2 are not satisfied
Note: An initial calibration must be done when new HRCC-3, sample fortification, or recovery std. soln. from another lot is used
D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7.8 Analysis
7.8.1 Reduce extract or blank volume to 10 or 50 uL
7.8.2 Inject 2 ul aliquot of the sample into the GC
7.8.3 Acquire SIM data according to Section 7.6.2 and 7.6.3
Note: Acquisition period must at least encompass PCDD/PCDF overall retention time window
7.8.4 GC Identification Criteria
.1 Relative Retention Times
.1 2,3,7,8 sub: Sample components relative retention time (RRT) w/in -1 to 3 seconds of retention time of labeled internal or recovery std.
.2 2,3,7,8 sub: Sample RRTs w/in homologous retention time windows if w/o labeled internal std.
.3 non 2,3,7,8 sub: Retention time w/in homologous retention time window
.4 Ion current responses for quantitation must reach maximum w/in 2 seconds
.5 Ion current responses for labeled stds. must reach maximum w/in 2 seconds
Note: Verify presence of 1,2,8,9-TCDD and 1,3,4,6,8-PeCDF in SICPs
.2 Ion Abundance Ratios
.1 Ratio of integrated ion current for two ions used for quantification w/in limits of homologous series
.3 Signal-to-Noise Ratio
.1 All ion current intensities > =2.5
.4 Polychlorinated Diphenyl Ether Interferences
.1 Corresponding PCDPE channel clear of signal > = S/N 2.5 at same retention time
7.9 Calculations
7.9.1 Calculate concentration of PCDD or PCDF compounds w/formula
7.9.2 Calculate % recovery of nine internal stds. using formula
Note: Add 1% recovery for human adipose tissue samples
7.9.3 Use smaller sample amt. if calculated concentration exceeds method calibration limits
7.9.4 Sum of isomer concentration is total concentration for a homologous series
7.9.5 Sample-Specific-Estimated Detection Limit (EDL)
EDL: Analyte concentration yielding peak ht. 2.5x noise level. EDLs calculated for non-identified 2,3,7,8-sub congeners
Two methods of calculation:
.1 Samples w/response <2.5x noise for both quantification ions
.1 Use EDL expression to calculate for absent 2,3,7,8 substituted PCDD/PCDF
.2 Samples w/response >2.5x noise for at least 1 quantification ion
.1 Calculate "Estimated Maximum Possible Concentration" (EMPC) when signal > 2.5x noise and retention time the same
7.9.6 Relative percent difference (RPD) formula
7.9.7 Calculation of 2,3,7,8-TCDD toxicity equivalent factors (TEF) of PCDDs and PCDFs
.1 Two GC Column TEF Determination: Reanalyze sample extract on 60 meter SP-2330 column
.1 Concentrations of specified congeners calculated from analysis done on DB-5 column
.2 Concentrations of specified congeners calculated from analysis done on SP-2330 column w/different GC/MS conditions
Note: Confirmation and quantification of 2,3,7,8-TCDD done on either column as long as Section 8.1.2 criteria met
.3 GC peak must meet criteria of Sections 7.8.4.2, 7.8.4.3, and/or 7.8.4.1.1 RRTs of 2,3,7,8-sub congeners w/no carbon-labeled analogues referred to w/in 0.006 RRT units of carbon-labeled std
Stop