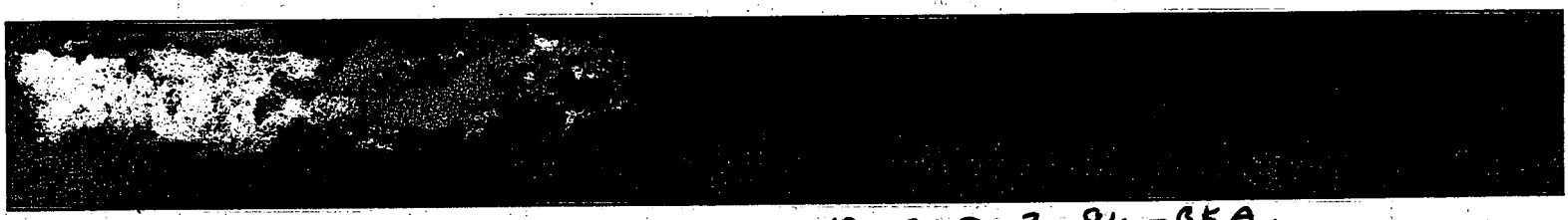

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INTRODUCTION

A considerable volume of recent environmental analytical chemistry research on polychlorinated dibenzo-p-dioxins (PCDD's) has been devoted to development of improved methods for the isolation and unambiguous identification of various isomers with special reference to 2,3,7,8-TCDD. In addition, increasing attention has been paid to increase the precision and accuracy during quantitative analysis of PCDD's and PCDF's at ultra trace levels (viz Table 1). The analytical scheme for quantitative analysis, for a given matrix, involves several pretreatment and cleanup procedures and those used depend upon the nature of the sample and the relative concentrations of interfering substances present in the sample.

In our laboratory, various methods of sample treatment, extraction and cleanup were evaluated in order to develop a suitable analytical scheme which would provide a selective determination of PCDD's by HRGC/ECD or HRGC/GRMS in a wide variety of environmental samples (1). These include natural waters, sediments, commercial chlorophenols, wood preservatives, wood shaving sprayed with wood

preservative, fish and fine grained sediment and other solid materials such as fly ash.

This report provides a detailed description of the methods developed during the past three years and is aimed at aiding the analyst to obtain reproducible results. The methods described below were used to generate analytical data during the thermal destruction study of chlorophenols (2) as well as those methods currently being employed by the Water Quality Laboratory (WQL) and the Great Lakes Fisheries Research Board (GLFRB) for the analysis of dioxins and furans. The methods for dioxins and furans are further applications of the techniques which were previously developed for PAH's (3) and carbamates (4).

Further improvement of the technique is continuing to expand the scope, precision and cost effectiveness and a more complete validation of the procedure is presently being carried out in conjunction with AMD Study 611. The summary of the technique is illustrated in Figure 1.

Table 1. Internal Standard (2 ng) Recoveries of 2,3,7,8-TCDD ^{37}Cl in Fish

Hc GPC/NA PO_4/Al O /CF — GC/EC detection; GC/MS confirmation

Fish Recoveries	
Sample	Internal Standard Recovery Percent
2,3,7,8-TCDD ^{1}Cl	82-89
MF1	60
MF2	74
MF4	65
MF5	72
MF6	64
TCDD #7	80

HcGPC = High capacity gel permeation chromatography utilizing styragel column.

1: Scope and Application

1.1: The proposed current methodology was tested using four tetra isomers, penta, hexa, hepta and octa chloro-dibenzo-p-dioxins and one isomer of tetra chloro-dibenzo-p-furan.

1.2: Fish tissues, commercial grade phenols, wood preservative formulations, water, sediment, flyash, gull and salmon eggs and samples from the thermal destruction of chlorophenols have been analyzed.

1.3: The method was extensively tested using labelled 2,3,7,8-TCDD with ^{13}C and ^{37}Cl isotopes for monitoring recoveries from fish tissues.

1.4: Other dioxins and furans can be determined by adjusting time frames and/or mobile phase composition along with delivery volumes associated with the various cleanup and isolation procedures.

1.5: Cleanup methodology may vary, depending upon sample type or nature of the matrix. The analytical detection limits will be affected by the nature of the matrix and degree of cleanup. Also the GC-MS detection limits are affected by the multiple ion monitoring associated with the spiked matrix technique.

1.6: Analysis by GC-MS is carried out utilizing a fused silica capillary column coupled directly with the ion source of the mass spectrometer. The system is capable of separating 13 isomers of TCDD.

2. Principle and Theory

2.1: Environmental samples are pretreated with 1N/HCl and subsequently extracted with mechanical agitation and toluene or soxhleted with hot toluene.

2.2: Resulting emulsions [associated with subsection (2.1)] are centrifuged and the organic layer spiked with labelled standard, washed with water and dried over Na_2SO_4 prior to concentration and subsequent preparative gel permeation chromatography using a commercially available styragel column.

2.3: The dioxin containing fraction from subsection (2.2) is treated with 0.05 M trisodium phosphate solution to remove trace phenolics and sulphur containing compounds. If lipid material is suspected to be remaining, the organic layer is washed with 50 mL of 10N H_2SO_4. The final solution is then washed with 100 mL deionized water and dried over Na_2SO_4 prior to concentration to dryness.

2.4: The sample is applied to an alumina column after reconstituting in hexane. The toluene fraction is collected and the solvent is changed to methylene chloride/cyclohexane 1:1 for the final carbon/fibre isolation.

2.5: The total sample dissolved in 0.9 mL of 1:1 methylene chloride/ cyclohexane is injected into the carbon/fibre system. The carbon/fibre module is then eluted with several solvent systems prior to the back elution with toluene. The toluene fraction is then concentrated to dryness and reconstituted in iso-octane.

2.6: Prescreening for the presence of dioxins and/or furans is achieved using HRGC with associated ECD and a DB-1 fused silica capillary column or equivalent.

2.7: Quantitative analysis is carried out with GC/MS utilizing a fused silica column capable of separating the various dioxin and furan isomers. The minimum detection limit is defined as the amount of 2,3,7,8-TCDD that provided clearly defined peak shapes at 320 and 322 masses and having the correct isotopic ratio (0.79:1 ± 10%) and with a signal-to-noise ratio greater than 2.5:1. It is possible to detect 5.0 pg using 3 µL injection volume.

3: Interferences

3.1: A number of organic compounds such as organo chlorines (DDE), PCB's, phthalate esters, toxaphene, phenols, sulphur containing components and high molecular weight substances such as lipids and humic and fulvic acids may interfere with this procedure. The extent of these matrix associated interferences will vary greatly depending on the species and age of a fish or the source and type of sediment and/or other samples. The cleanup and isolation procedures in section 2 can overcome most of these interferences. However, some samples may require additional cleanup steps using micro-bore and/or conventional HPLC techniques.

3.2: Method interferences may be introduced by the solvents, reagents, glassware, gases and other equipment used in processing the sample.

3.3: Dioxins are sensitive to UV light degredation and therefore the work area should be free of UV light.

4: Safety Precautions

4.1: The toxicity of TCDD has not been clearly defined, however, dioxins and furans substituted in the 2,3,7 and 8 positions are

known for their high carcinogenicity. 2,3,7,8-TCDD is suspected of being the most toxic man-made substance. Handling, therefore, must be carried out with extreme care to avoid skin contact and inhalation or ingestion.

4.2: Each agency or laboratory should restrict work to specified areas equipped with fumehoods and/or exhaust equipment.

4.3: Laboratory personnel must be made aware of safety precautions and provided with protective clothing and equipment. TCDD has been handled safely in analytical and biological laboratories utilizing the technique commonly used for handling radioactive and infectious materials.

4.4: Solid wastes must be "bagged" and saved for safe disposal. Liquid waste must be saved in proper containers and can be allowed to slowly evaporate in a good exhaust hood. Residue should be dissolved in methanol or ethanol and UV irradiated for 2 - 3 days. (Use F 40 BL lamps or equivalent).

4.5: Gas chromatographs should have their exhaust gases vented through charcoal filters straight to the atmosphere.

5: Sampling Procedure and Storage

5.1: Whole fish may be frozen in clean aluminum foil and later thawed and thoroughly homogenized prior to analysis. Residual fish tissue may be refrozen in amber glass bottles. Tissue that has been frozen must be homogenized again when thawed to redistribute the lipids which migrate to the top of the container.

5.2: Sediment samples can be frozen in metal containers and homogenized prior to analysis or can be freeze dried and stored in amber bottles for later analysis.

5.3: Flyash samples can be stored in amber bottles.

5.4: Egg type samples can be homogenized and stored frozen in amber bottles.

5.5: Water and other fluid samples can be stored in sealed bottles.

6: Apparatus

6.1: Centrifugation Apparatus

- 250 mL steel centrifuge cups.
- centrifuge capable of accommodating 250 mL cups and 4500 - 5000 rpm.

6.2: Filtering Apparatus

- 5 mL gas tight syringe, with Luer Lock.
- Nylon 66, disposable filter unit, 25 mm, 0.2 µm porosity.

6.3: Glassware

- 500 mL Erlenmayer flasks with 23/40 stoppers.
- 500 mL separating funnels.
- Allihn filters with 4 cm x 10 cm reservoir and sintered glass discs of B porosity.
- 500 mL, 300 mL and 100 mL round bottom flasks with 24/40 stoppers.
- micro syringes of various sizes to cover the range 10 µL to 500 µL.
- graduated 15 mL centrifuge tubes with stoppers.
- disposable glass pipettes.
- chromatographic columns 2.5 cm I.D. equipped with teflon stopcock.

6.4: Concentration Apparatus

- Buchii rotary evaporator with vacuum monitor and temperature controlled water bath.

Searle vortex evaporator, vortex action should be adjusted according to volume and the temperature adjusted to 35°C. (Reduce temperature for $CHCl_3$ and the more volatile solvents).

6.5: Extraction Apparatus

- Willems polytron homogenizer with model PT10 generator or equivalent.
- Soxhlet apparatus, of appropriate size to accommodate 10-25 g sample size in glass thimbles containing fritted discs of porosity to facilitate percolation of solvent through the sample.
- Bronson model 350, cell disruptor.

6.6: Gel Permeation Liquid Chromatographic System

- Valco or suitable injection valve.
- 5 mL syringe (gas tight).
- 5 mL sample loop.
- 60°A Styragel column.
- UV detector with 254 nm filter for monitoring the column effluent.
- solvent delivery system (pump) capable of constant flow with medium to low back pressure.

6.7: Alumina Column Cleanup Apparatus

- disposable pasteur pipettes.
- chromatographic column 2.5 cm I.D. with teflon stop cock (no fritted disc).

6.8: Carbon-Fibre Isolation Apparatus

- solvent delivery system capable of constant flow at low back pressures.
- solvent selector valve allowing selection of 5-6 solvents.
- fraction collector and controller.
- 6 port switching valve equipped with 1 mL sample loop.
- 1 mL syringe.
- 4 mm I.D. glass tubes 7.2 cm long, previously annealed and fitted with zero dead volume fittings and 2 µm stainless steel frits.
- associated tubing and connections.

6.9: Gas Chromatography Detection System

- capillary gas chromatographic system equipped with electron capture detector.
- capillary column (DB-1 or DB-5) for TCDD prescreening.
- splitless, injection technique.

6.10: GC/MS Detection System

- Finnigan model 4000, upgraded with a 4500 ion source and associated datapak.

splitless injection system with an SP2340 60 m fused silica capillary column.

7: Reagents

7.1: All reagents must be pesticide grade or better and must be checked prior to use.

7.2: Trisodium phosphate (reagent grade).

7.3: Hydrochloric acid (reagent grade).

7.4: Water - Millipore deionized or equivalent.

7.5: Toluene - distilled in glass.

7.6: Chloroform - distilled in glass.

7.7: Hexane - distilled in glass.

7.8: Alumina - neutral, heated at least 12 hours at 130°C then desiccated before use.

7.9: Methylene Chloride - distilled in glass.

7.10: Cyclohexane - distilled in glass.

7.11: Glass wool - silanized and solvent extracted.

7.12: Carbon - Amoco Px-21.

7.13: Sulphuric Acid - Reagent grade.

7.14: TOYO glass fibre filter papers - type GA 200.

7.15: Ethyl Acetate - distilled in glass.

7.16: Benzene - distilled in glass.

7.17: Iso Octane - distilled in glass.

7.18: Alumina-neutral, Brockman activity I 80-200 mesh, heated overnight at 550°C, cooled and deactivated with 1% water and allowed to tumble whole day.

7:19 Sodium Sulphate - anhydrous, heated to 650°C for a minimum 12 hours.

8: Concentration and Extraction Techniques

8.1: The choice of extraction technique depends upon the type of sample and/or its makeup. Improved recoveries of dioxins in fish tissue, sediments of organic nature and egg samples have been found when the samples are pretreated with 1N HCl and extracted with toluene overnight on a mechanical agitator. Sediments of high sand or grit content may be sonified and particulates such as flyash must be Soxhlet extracted, after a pretreatment with 1N HCl and filtered. The solid is then mixed with Na_2SO_4 prior to extraction and the filtrate is liquid/liquid extracted. Water samples are solvent extracted.

8.2: Extraction of Fish Tissues

8.2.1: Homogenize the sample using a polytron or blender.

8.2.2: Subsample 10-20 g of sample into a 500 mL Ehrlenmayer flask.

8.2.3: Add 100 mL of 1N HCl and 100 mL of toluene to the flask and place on wrist shaker or equivalent overnight, ensuring sufficient agitation for complete mixing of the contents.

8.2.4: The presence and/or intensity of the resulting emulsion determines the analytical procedure to be followed.

8.2.5: A light emulsion (see Operational Note 1), in which some separation occurs, allows the organic layer on top, simply to be decanted into the separatory funnel and the aqueous layer and solids to be polytroned in the reaction vessel with additional toluene. A medium emulsion (see Operational Note 2) requires transfer to a separatory funnel and allowing the layers to separate then running the solids and lower aqueous layer into the stainless steel cups followed by Polytron homogenization with toluene.

A dense, thick type of emulsion (see Operational Note 3), is transferred with any solids to the centrifuge cups immediately, and then centrifuged at 4000 - 4500 rpm for 15 minutes. Content of the cup is then transferred to the separatory funnel and allowed to settle and separate. The lower aqueous layer and any solids are run back into the centrifuge cup and polytroned with additional toluene.

This alternate separation, polytron homogenization and centrifugation technique is repeated twice with 100 mL toluene each time, after the initial separation of the toluene has been accomplished.

8.2.6: The toluene extract is washed once with 100 mL of millipore deionized water and then dryed by passing through a 2.5 - 3.0 cm plug of Na_2SO_4 contained in an Allihn filter. The dried extract is collected in a 500 mL round bottom flask for GPC cleanup.

8.3: Extraction of Sediments

8.3.1: Sediments of an organic nature are extracted similarly to fish tissue, however, if there is sand and/or grit present, the extraction is carried out using the cell disruptor au lieu de polytron homogenization after the preliminary 1N HCl treatment.

8.3.2: Freeze dried sediments are stirred with 1N HCl for one hour. The solution is then filtered by suction. The solids are ground with Na_2SO_4 until a free flowing mass is obtained and then it is charged into the glass thimble and soxhlet extracted for 18 hours. The acid filtrate is extracted with toluene.

8.4: Extraction of Flyash

8.4.1: Flyash, which exhibits strong absorption of dioxin and furans is treated with 1N HCl for one hour and then filtered. The solids are then ground with Na_2SO_4 until a free flowing mass is obtained and then it is soxhleted for 36 hours with hot toluene. The acidified filtrate is extracted with toluene.

8.5: Extraction of Woodshavings

8.5.1: Wood shavings containing wood preservatives are treated by the same procedure as organic type sediments.

8.6: Extraction of Egg Samples

8.6.1: Egg samples give heavy emulsions in the extraction procedures and are handled by the same procedure as fish tissue.

8.7: Extraction of Water Samples

8.7.1: Water samples are extracted with methylene chloride or toluene (toluene gives less emulsion problems). Depending on the source and nature of the sample and its extract, the cleanup procedure can be reduced accordingly.

9: Cleanup

9.1: The cleanup technique used will vary with type and nature of the sample to be analyzed.

9.2: Cleanup by Gel-Permeation Chromatography (GPC).

9.2.1: Concentrate the original toluene extract in the 500 mL round bottom flask to dryness until the organic solvent is no longer distilled over on the rotary evaporator.

9.2.2: Transfer the residue (lipids in the case of fish) to 15 mL centrifuge tubes with chloroform. Adjust the volume of sample so that 0.5-1 g of sample residue/1 mL of chloroform is not exceeded. In some cases the complete transfer of lipids may require additional solvent.

9.2.3: Filter all samples, through a prewashed Nylon 66 filtering apparatus, utilizing a 5 mL gas tight syringe (see Operational Note 4).

9.2.4: Inject 4.5 mL into the GPC system operated with chloroform at 5 mL/min flow and collect fraction 40-55 minutes for

2,3,7,8=TCDD (see Table 2) for the elution order of various organic compounds using Hc GPC).

9.3: Cleanup - Trisodium Phosphate removal of phenols, sulphur and acidic compounds.

9.3.1: Transfer fraction 30-41 from the GPC cleanup to a 250 mL separation funnel and treat once with 100 mL 0.05 $Na_3PO_4.12\ H_2O$ for 2 min. Wash the organic layer with 100 mL deionized water.

9.3.2: Dry the chloroform extract by passing it through a 2.5 - 3.0 cm plug of Na_2SO_4 contained in an Allihn tube and collect in a 300 mL round bottom flask.

9.3.3: Concentrate chloroform extract on the rotary evaporator to dryness.

9.4: Cleanup - Sulphuric acid removal of residual lipids and basic compounds (see Operation Note 5).

9.4.1: Treat the washed chloroform extract from subsection (9.3.1) with 50 mL of 10N H_2SO_4 for 2 min. Wash the organic layer once with 100 mL of deionized water.

9.4.2: Dry through sodium sulphate as before and collect in a 300 mL round bottom flask.

9.4.3: Concentrate on the rotary evaporator to dryness and save for alumina column cleanup.

Table 2. Elution Order of Various Organic Compounds
Column: Styragel, Particle Size 37-75, Permeability Range 60Å
Mobile Phase - Chloroform Flow Rate = 5 mL/min

Compound	Fraction Time (min)
Polynuclear Aromatic Hydrocarbons (PAH)	48 - 56
Phenol	56 - 65
Pentachlorophenol	45 - 51
Phthalate Esters	
• DEHP	31 - 37
• DBP	33 - 40
• Dnonyl P	30 - 39
• DEP	36 - 44
PCBs	43 - 51.25
Humic and Fulvic Acids	25 - 32
Lipids and Esters from Fish Concentrate	25 - 44
1,2,3,4-TCDD	
2,3,7,8-TCDD	
Penta, hexa, hepta and ocata chlorodibenzo-p-dioxins	40 - 54

The individual fractions for various classes of compounds may vary depending upon sample, flow rate, pressure, etc. Therefore, it is recommended to standardize the column using fluoranthene and select appropriate elution window to cover compounds of interest.

9.5: Cleanup by Alumina Column Chromatography:

9.5.1: Reconstitute the residue from (9.3.3) or (9.4.3) in 1 mL of hexane.

9.5.2: Apply the 1 mL hexane extract to the top of a 5 cm Al_2O_3 column contained in a disposable pipette that has previously been eluted with 7 mL hexane.

9.5.3: Wash the sample vessel with hexane and apply to the alumina column until 10 mL of eluant has been collected. Discard.

9.5.4: Elute the column with 10 mL of toluene into 15 mL centrifuge tube and save for carbon-fibre cleanup and isolation.

Note: If samples are suspected of containing large amounts of interferences, a large scale alumina column may be employed. (See Operational Note 6).

9.6: Carbon Fibre Cleanup and Isolation

9.6.1: Concentrate the toluene fraction from the alumina column cleanup step to dryness and then redissolve the residue in 1 mL of 1:1 methylene chloride/cyclohexane.

9.6.2: Inject the whole sample extract into the carbon fibre system (see Operational Note 7) and elute in succession as follows:

eluant 1: 44 mL 1:1 methylene chloride/cyclohexane

eluant 2: 30 mL ethyl acetate

eluant 3: 40 mL 10% Benzene in ethyl acetate

eluant 4: 35 mL 50% Benzene in ethyl acetate

eluant 5: 30 mL toluene

Note: Volumes can be adjusted as required.

9.6.3: Concentrate the toluene fraction collected in 100 mL round bottom flask just to dryness and transfer to a 15 mL centrifuge tube with hexane.

9.6.4: Concentrate the hexane to dryness on the vortex evaporator and then adjust the volume of ISO octane for GC and GC/MS analysis.

10: Qualitative Analysis using HRGC-ECD

10.1: Prescreening of the final iso-octane extract is accomplished using a Hewlett-Packard 5880 gas chromatograph in the splitless mode and equippped with a DB-1 capillary column connected to the ECD. Conditions are:

Inj. port 250°C	Initial hold 3 min
Detector 300°C	Program 30°C/min to 220°C
Oven Profile	Program 4°C/min to 260°C
Initial temperature 80°C	Final hold 30 min

10.2: Because the sample is spiked with ^{37}Cl or ^{13}C labelled isotopes of 2,3,7,8-TCDD, both of which co-elute with native 2,3,7,8-TCDD, quantitation is impossible.

10.3: Some indication of recovery may be possible by comparison to absolute standards, however, values attributed to native and labelled compounds cannot be distinguished.

11: Quantitation and Qualitation by Low Resolution Mass Spectrometry:

11.1: Quantitation and qualitation is achieved with the Finnigan 4000 low resolution mass spectrometer upgraded with a 4500 ion source and associated data pak. The gas chromatograph is equipped with a 60M SP2340 fused silica capillary column coupled directly to the ion source.

A Hewlett Packard capillary injector was used in the splitless mode employing a 30 second vent time. The operating conditions are summarized below:

Injector 260°C	Initial hold 2 min
Helium carrier gas at 20 psi	Program at 15°C/min to 280°C
Initial oven teperature 80°C	Final hold 10 min

MS Conditions:

EI	Electron Multiplier 1300 volts
Transfer line @ 270°C	Source 250°C
70 EV	Manifold 100°C
Emission current 0.32 MA	Scan 5 ions (257, 320.322, 328, 332) - .6 to 1 sec/scan

11.2: Qualitative analysis requires that retention times of the standard and sample agree within ± 1.0% and the m/z ratio for 320:322 native 2,3,7,8-TCDD in the sample must be 0.8 ± 10% for positive identification.

11.3: Because the analysis is done in the Multiple Ion mode (5) the detection limit is reduced to approximately 5 pg.

11.4: Quantitation is achieved, using standard addition technique, by comparing the peak areas of the m/z 257,320, or 322 peak of the native 2,3,7,8-TCDD in the sample, the m/z 328 peak of the ^{37}Cl or the m/z peak at 332 for the ^{13}C labelled standards that have been spiked at a known level in the sample.

11.5: Recoveries of the 2,3,7,8-TCDD can be determined by comparing the spiked labelled ^{13}C or ^{37}Cl peak areas in the sample extract to a known external standard.

12: Recovery:

12.1: The recovery of 2,3,7,8-TCDD ^{37}Cl from six fish tissue samples of varying lipid content ranged from 75-82%. The recovery of standard carried through the procedure without the sample matrix proved to be 85%.

13: Operational Notes

13.1: Vigorous agitation of a sample, with an organic solvent will almost always produce an emulsion. A light emulsion will resolve itself upon allowing the sample vessel to stand.

13.2: A medium emulsion will require some assistance for disposal. Often a slight agitation of the sample vessel is adequate, however, some standing time will be necessary.

13.3: A dense emulsion usually has a milky appearance which indicates the layers (contents of sample vessel) will not separate.

13.4: The Nylon 66 filtering apparatus and associated syringe must be prewashed with 15-20 mL of chloroform prior to use.

Samples may be transferred from the round bottom flask used for concentration, directly to the syringe for filtration into a 15 mL centrifuge tube. Avoid unnecessary dilution of sample because concentration at this point can be problematic due to the consistency of the sample extract.

13.5: Excessively contaminated samples require an acid wash to remove the remaining biogenic material in order to facilitate the fractionation of the carbon-fibre system. Concentration of the chloroform extract prior to the application of the sample to the alumina column will provide the analyst with the necessary indications.

13.6: Fish tissue of the salmon or trout family and sediment type samples require the use of a 30 g alumina cleanup column. Neutral alumina Brockman activity 1 heated overnight to 550°C deactivated with 1% water and tumbled for one day is packed into one 2.5 cm chromatographic column and topped with a small amount of Na_2SO_4. Apply the sample, with 1% methylene chloride in hexane, elute with 200 mL of same and discard. Remove TCDD with 80 mL of toluene.

13.7: The carbon-fibre is prepared by shredding 600 mg of glass fibre filters in 100 mL of methylene chloride using a polytron or equivalent. Add 50 mg AMOCO PX-21 carbon and stir the mixture until the carbon is evently distributed.

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EXTRACTION, CLEAN-UP, ISOLATION AND QUANTITATION OF DIOXINS AND FURANS FROM FISH TISSUE