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CHARACTERISTICS OF AN EXTRACTION AND PURIFICATION PROCEDURE FOR CHLORINATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS IN SOIL AND LIVER

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SUMMARY

Liver is extracted with chloroform–methanol to give essentially quantitative transfer of endogenous chlorinated dibenzo-*p*-dioxins (CDDs) and dibenzofurans (CDFs) into the organic phase. A new procedure involving LH-20 Sephadex is used to remove most of the lipids from the extract. Soil is extracted by a simple, rapid and economical procedure giving very high recoveries of CDDs and CDFs from sandy soil, various types of clay, and humus-rich loam. Subsequent cleanup on basic and acidic alumina complete the preparation for gas chromatography-mass spectrometric analysis. The use of propylene glycol as a "keeper" and of 2,3,7-trichlorodibenzo-*p*-dioxin as a carrier minimizes losses during evaporation of solvents and on glass surfaces. Interactions of 2,3,7,8-CDD with organic material in loam slightly reduce recovery but there is no indication of high affinity binding sites, the losses being apparently associated with simple distribution coefficients. Special precautions needed to avoid losses of CDFs on alumina chromatography are described, and the effect of "aging" spiked soil is discussed.

INTRODUCTION

Chlorinated dibenzo-*p*-dioxins occur as undesirable side products during the synthesis of products derived from halogenated phenols[1,2] as well as from a variety of combustion processes[3,4]. They are found in lumber treated with pentachlorophenol[5–7], in fish from polluted waters[8,9], and, at extremely low levels, in human tissues in spite of inability to identify a source of exposure[10]. The related family of chlorinated dibenzofurans occurs as a contaminant of the polychlorinated biphenyls formerly used as transformer fluids, heat exchangers and dielectrics[11,12]. The dibenzofurans are also formed during combustion processes[4,13].

Both families of aryl cyclic ethers include members having extreme toxicity to animals, with the 2,3,7,8-tetrachloro isomers being much more toxic than most of the others[14]. The more unsymmetrical isomers, as well as the non-halogenated and

fully halogenated members, have very low degrees of toxicity[14]. The most toxic compound of either family, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (LD_{50} of 2 μg/kg in the guinea pig, ref. 15), has in the past been of much more concern to toxicologists and environmentalists than the other members of these families of compounds. Because of the extreme toxicity of this compound, analytical methods capable of conclusive identification and quantitative measurement at the parts-per-trillion (ppt) level have been considered necessary for its determination. Although methods effective at the parts-per-billion (ppb)* level have been developed based on such varied techniques as radioimmunoassay[16], induction bioassay[17], gas chromatography with electron-capture detection[18], and electron-spin resonance spectroscopy[19], methods considered reliable at the low ppt level have been based primarily on gas chromatography–mass spectrometry (GC–MS)[10] or mass spectrometry–mass spectrometry[20].

More recently, it has become clear that there is a need for methodology to permit quantitative determination of the complete spectrum of chlorinated dibenzo-*p*-dioxin (CDD) and dibenzofuran (CDF) isomers present in samples, though not necessarily at the one ppt level. Several of these compounds other than the 2,3,7,8-tetrachloro isomers show significant degrees of toxicity[14]. Moreover, the particular mixture of isomers present in a given sample provides clues as to the source or origin of the contamination[21].

The very extensive cleanup or enrichment needed to avoid qualitative[22,23] or quantitative[24] interference in these ppt level analyses has led to the development of a variety of sample workup procedures. Many of these procedures are optimized for the recovery of 2,3,7,8-CDD and less acceptable if not totally unsuitable for compounds having more or fewer than four chlorines. Procedures capable of giving acceptable recoveries of CDDs have in most cases not been validated for CDFs. Frequently, methods have been developed and tested using fortified ("spiked") surrogate samples, with no evidence provided to assess whether or not recovery of the "spiked" should be considered a valid measure of recovery of endogenous CDD or CDF.

In what follows we describe our efforts to develop extraction and cleanup procedures for CDDs and CDFs in general, in soil and liver matrices, with high and reproducible recoveries, and with a relatively rapid achievement of an adequate elimination of interferences to permit reliable determination by GC–MS at below ppb levels.

EXPERIMENTAL

GC–MS was performed as described previously[25,26] using a VG ZAB 2F mass spectrometer coupled to a Hewlett-Packard Model 5700 gas chromatograph. Bonded methyl silicone phase fused-silica capillary columns were obtained from J&W Scientific (Rancho Cardova, CA, U.S.A.). Radioactivity in extracts and open column fractions was measured by liquid scintillation counting with a Packard Tri-Carb scintillation spectrometer. Tissues were digested in NCS tissue solubilizer (Amersham, Arlington Heights, IL, U.S.A.) and radioassayed in Riafluor scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.). Non-aqueous samples were radioassayed in

* Throughout this article the American billion (10^9) and trillion (10^{12}) are meant.

Liquifluor (New England Nuclear); counting efficiencies were determined from standard curves relative to the external standard count ratio[27].

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin with one ring uniformly labeled with ^{14}C (148 μCi/μmole) was obtained from KOR Isotopes (Cambridge, MA, U.S.A.) as was the corresponding compound >98.7% enriched with ^{13}C. Uniformly ^{14}C-labeled biphenyl was chlorinated to 54% chlorine, giving a product distribution analogous to Aroclor 1254. Unlabeled Aroclor 1254 was tritiated under pressure (Wilzbach procedure[28]); since some dechlorination occurs, the product more nearly resembled Arochlor 1248 in isomer distribution. 3,3',4,4'-[U-^{14}C]tetrachlorobiphenyl was kindly provided by Dr. H. B. Matthews of this institute. A series of non-radioactive CDD and CDF standards (described in ref. 16) was provided by Dr. John Moore (currently of the U.S.E.P.A.). A mixture of CDFs (3–6 chlorines per molecule) was produced by chlorination of [U-^{14}C]dibenzofuran, initially 138 μCi/μmole. Other common environmental pollutants were from sources listed previously[29].

Two types of chromatographic alumina were used in this study. Basic alumina was A-540, 80–200 mesh, from Fisher Scientific, Raleigh, NC, U.S.A. Acidic alumina was grade 90, 70–230 mesh, EM Labs., Elmsford, NY, U.S.A. All alumina was activated at 130°C for at least 16 h but not more than 72 h before use[30]. Chromatographic and extraction solvents, including chloroform, methanol, *n*-hexane, methylene chloride, ethyl acetate and acetone, were either Omnisolve (Preiser Scientific, Durham, NC, U.S.A.) or glass-distilled pesticide grades (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), and were the most stringently controlled grades available from these sources. Methylene chloride and *n*-hexane were routinely stored over molecular sieves 13-X and Davidson grade 05 silica gel respectively, to ensure dryness. Sephadex LH-20 was obtained through Sigma, St. Louis, MO, U.S.A., washed with and stored as a slurry in methanol. Anhydrous sodium sulfate was pesticide grade (J. T. Baker); it and ignited sand were obtained from Fisher Scientific.

Open column chromatography was performed in tubes equipped with reservoir bulbs and Teflon® stopcocks. A glass wool plug was placed at the bottom of each tube, adsorbant (alumina) was added dry or LH-20 added as a slurry in methylene chloride–methanol (1:1, v/v), the column was allowed to form with gentle tapping (alumina) or gravity flow (LH-20), a 1-cm layer of anhydrous sodium sulfate (for alumina columns) or sand (LH-20) was used to top the columns, and all separations were at room temperature. Samples were loaded directly onto dry alumina columns with stopcocks open. Pre-washing with an organic solvent always resulted in broadened peaks and poor separation, so was avoided. If the alumina needed a prewash, that was done with methylene chloride prior to the 130°C activation.

Liver samples were taken from Sprague-Dawley (CD strain, Charles River) or Fischer 344 rats, blotted, weighed and processed fresh. Soil types included (1) a mixed loam from sites in Indiana, (2) a sandy woods soil from the Piedmont area of North Carolina, (3) nearly pure red clay, predominately kaolinite, from North Carolina, (4) pure quartz sand from Ontario, Canada, and (5) bentonite clay from Wyoming. Characteristics of these soils will be discussed later. In all cases the soils were screened to pass 40 mesh and equilibrated to a relative humidity of 50% before use.

Extraction

Liver. This procedure is essentially as was described previously[30]. Liver tissue

(0.5–10 g) is spiked with 0.1 μg of 2,3,7-trichlorodibenzo-*p*-dioxin to serve as a carrier, and with ^{13}C-enriched 2,3,7,8-tetrachlorodibenzo-*p*-dioxin at 100 pg per g tissue as internal standard, both dissolved in 10 μl of dimethyl sulfoxide (DMSO). The tissue is then homogenized (Waring blender) with 20 volumes of chloroform–methanol (2:1, v/v). The homogenate is filtered with suction through glass fiber filter paper (Reeve Angel grade 934AH or equivalent). The filter paper and cake is re-blended with half as much chloroform–methanol as was used originally, the second homogenate is again filtered through glass fiber filter paper, and the filtrates are combined in a separatory funnel. To the extract are added 5.2 ml of 1.2% aqueous potassium chloride per gram of liver processed. The phases are mixed, let clear, and the lower phase collected in a round bottom flask. Solvent is removed by rotary evaporation under aspirator vacuum at 35–40°C (no higher).

Soil. An abbreviated description of this technique has been given previously[31]. A glass wool plug is placed in the bottom of a 125-ml separatory funnel equipped with a Teflon stopcock. Ten grams of anhydrous sodium sulfate are layered over the glass wool, then 20 g of soil added, and finally another layer of 10 g of sodium sulfate is added to top the "sandwich". The dry material is saturated with 14 ml of acetone, which is just enough to wet all of the solids without dripping through and which contains both trichlorodibenzo-*p*-dioxin carrier and internal standard. The sample is then elutriated with 50 ml of ethyl acetate, and when dripping stops, with 100 ml of methylene chloride. All operations are at room temperature. Many solvents and solvent combinations have been tried; none were as effective as that described above. The combined effluent is rotary evaporated under aspirator vacuum at 35–40°C in a round bottom flask. No residual water should be present, but if moisture is observed, methylene chloride should be added to the flask and evaporated to eliminate residual moisture.

Sample fortification

Since CDDs and CDFs tend to accumulate in liver *in vivo*[32], and since we have never detected radioactive metabolites (alteration products) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in liver following oral administration of the ^{14}C-labeled compound to rats or guinea pigs (unpublished observations), in most cases we generated endogenous rather than spiked liver samples containing ^{14}C-labeled 2,3,7,8-CDD. Corn oil containing the labeled CDD was administered intragastrically by gavage, and the rats sacrificed with carbon dioxide at various times thereafter. Non-radioactive CDDs and CDFs were spiked into liver in DMSO during sonication as described previously[31].

Soil was spiked by adding a solution of test compound in 3 ml of acetone to 20 g of soil, allowing the acetone to evaporate in a fume hood overnight. The soil was then tumble-blended in a glass-stoppered flask, which was subsequently sealed with Teflon tape until processed. In some cases the spiked soil was held in open containers, protected from light, for up to 30 days at room temperature prior to extraction. The specified volume of acetone, 0.15 ml per g of soil, was optimum for uniformity. Lesser amounts did not permeate all of the soil, while greater amounts left test compound coated on the walls of the flask.

Cleanup procedure

The dried residue from extraction of liver was dissolved in a minimal volume of methylene chloride–methanol (1:1, v/v) and transferred to the top of a column (1.5 cm diameter) containing 20 g of LH-20 Sephadex in the same solvent mixture. The column was eluted with this solvent mixture, discarding the first 70 ml to elute (lipids and other aliphatics), collecting the next 50 ml (total aromatics), and washing with a final 50 ml to ensure that the column was clean for subsequent re-use. This column can be used for sequential samples until the top one cm of Sephadex becomes discolored, at which time the packing should be discarded.

To the fraction designated total aromatics were added 10 μl of propylene glycol as a "keeper". This treatment permitted rotary evaporation of the solvent at 40°C with minimal loss of even the more volatile polychlorinated biphenyls[29] and no detectable loss of CDDs or CDFs. Nor did it interfere with subsequent adsorption chromatography.

We have not found the LH-20 step necessary for extracts of soil, as in general the soil types we have had occasion to process contained extremely low levels of extractable, aliphatic organic material.

Either the dried residue from the total aromatics fraction from LH-20 (in the case of liver) or the dried residue from the original extraction (soil) was treated with 10 ml of *n*-hexane, which was again rotary evaporated to ensure the absence of traces of polar solvents. Severe losses of CDFs can occur in an unpredictable manner if this step is omitted. The residue is then taken up in 2 ml of *n*-hexane–methylene chloride (98:2, v/v), and loaded onto a column containing 3 g of activated A-540 alumina. The height-to-diameter ratio of this column is not critical. Fraction A is eluted with 30 ml of *n*-hexane–methylene chloride (98:2, v/v), and contains such components as chlorinated benzenes, naphthalenes, biphenyls, and diphenyl ethers[29]. The CDDs and CDFs are eluted together with 30 ml of *n*-hexane–methylene chloride (80:20, v/v) after which more polar materials (such as phthalate esters) may be eluted if desired. The CDD + CDF fraction is rotary evaporated at not over 25°C, and then redissolved in not over 2 ml of *n*-hexane–methylene chloride (99:1, v/v). This is loaded onto a column containing 2 g of acidic alumina, and washed with 20 ml of the same solvent to remove those materials, including the more polar polychlorinated biphenyls such as the 3,3′,4,4′-tetrachloro isomer, which "tail" badly on basic alumina. The CDD + CDF fraction is again eluted with *n*-hexane–methylene chloride (80:20, v/v), 20 ml, and rotary evaporated at room temperature. This final residue is transferred, using the minimum amount of methylene chloride, to a 1-ml Reactivial and blown just to dryness under a gentle stream of nitrogen at room temperature. The vial is sealed with a Teflon-lined screw cap and stored refrigerated (and dark) until analyzed. The final evaporation step requires the presence of the trichlorodibenzo-*p*-dioxin carrier, which seems to result in a residue that does not form a dust and escape.

For analysis, the residue is dissolved in a few microliters (usually 15) of either toluene or benzene. The use of hexane or isooctane leaves from 15 to 50% of 2,3,7,8-CDD or octachloro-CDD left on the glass walls.

RESULTS

Chromatography

The loss of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin into the 2% methylene chloride fraction during chromatography on A-540 alumina as a function of acetone content of the column loading solvent is shown in Table I. A significant amount of polar solvent may remain with the residue from rotary evaporation of the sample extract. Addition of hexane to the (apparently) dry residue, followed by another evaporation step, eliminates this source of variability in recovery. The CDFs are even more likely to inadvertently elute too soon than are the CSSs, but we have found A-540 alumina less likely to "leak" CDFs than any other commonly available grade of chromatographic alumina (unpublished observations). The only CDD or CDF that was not quantitatively eluted by 10 ml of 20% methylene chloride per g of A-540 alumina was octachloridibenzo-*p*-dioxin. This compound binds unusually tightly to alumina and we obtained only a mean recovery of 88% of a 10-ng load on a 3-g column with 30 ml of hexane–methylene chloride (80:20). In contrast, we do not observe "tailing" of any of the CDDs or CDFs on the acidic alumina column.

TABLE I

EFFECT OF A POLAR SOLVENT (ACETONE) ON RECOVERY OF 2,3,7,8-CDD FROM A-540 ALUMINA

Five 3-g columns of A-540 alumina were loaded with 9 ng of ^{14}C-labeled 2,3,7,8-CDD in hexane–methylene chloride (98:2, v/v) and eluted with 30 ml of the same solvent containing various amounts of acetone as indicated.

Acetone (%)	*CDD in fraction 1 (%)**
0	0.9
0.1	0.6
0.25	45.5
0.5	98.4
1	97.9

* Percentage of the ^{14}C loaded that was eluted "too soon", that is, in the fraction normally containing low polarity contaminants (biphenyls, diphenyl ethers, naphthalenes, etc.).

The preliminary cleanup step involving LH-20 Sephadex has not been described previously, so it was necessary to establish the elution characteristics of a variety of classes of compounds under the described conditions. Some of the relevant findings are summarized in Table II. Aromatics did not chromatograph on a molecular size basis, gave symmetrical peaks whose breakthrough volumes were independent of concentration, and could be isolated with about the same degree of removal of aliphatic contaminants (approx. 95%) as is usually achieved by partitioning against sulfuric acid[30]. Of the common lipids, only free fatty acids chromatographed slowly enough to overlap into the aromatics fraction to a significant extent. Removal of lipids on this column greatly reduces the size of the A-450 alumina column needed in the next step, as simple lipid monoesters such as cholesterol esters coelute with CDDs on alumina[30].

TABLE II

CHROMATOGRAPHIC PROPERTIES OF VARIOUS COMPOUNDS ON LH-20 SEPHADEX* IN METHYLENE CHLORIDE–METHANOL (1:1)

Compound or class	*Breakthrough volume (ml)***	*Peak elution volume (ml)*	*Percentage in fraction 2****
Triolein	37.5	46.5	<1
Cholesterol oleate	37.5	46.0	<1
Oleic Acid	48.0	70.2	50
Rat liver phospholipids	42.5	61.0	7
Medicinal mineral oil	38.0	48.2	5
Dibenzo-*p*-dioxin	61.0	80.0	95
Aroclor 1254	65.0	84.3	98
Anthracene	76.1	86.0	100
2,3,7,8-CDD	75.5	88.5	100
2,3,7,8-CDF	75.5	88.5	100
Octachloro-CDD	74.0	91.5	99
Perylene	92.5	95.2	100

* 20 g dry weight.
** Breakthrough = volume needed to elute the first 1% of the compound.
*** Fraction 1 = first 70 ml; fraction 2 = subsequent 50 ml.

Extraction

Liver. Since in general low levels of CDD and CDFs can not be quantified accurately by GC–MS in extracts of soil or tissues without some degree of preliminary cleanup, we could only measure extraction efficiencies for those compounds (2,3,7,7-CDD and -CDF) available in radioactive forms. In all cases tested, 2,3,7,8-CDF was extracted as completely as or more completely than 2,3,7,8-CDD, so only the latter will be discussed here. The specific activity of the ^{14}C-CDD, approximately 1000 dpm per ng, limited the concentrations that could accurately be measured in rat liver to $\geqslant 1$ ppb. In all cases considered here, liver was allowed to accumulate 2,3,7,8-CDD *in vivo,* and the levels present determined by radioassay of liver pieces as digests in NCS tissue solubilizer, the solubilization being performed directly in liquid scintillation vials. This permitted a direct comparison with amounts of ^{14}C-CDD extracted by the described procedure from other portions of the same liver. Results from several rats are summarized in Table III.

Recovery was independent of concentration over the range tested (10–1000 ppb). The time elapsed between exposure and sacrifice (up to 70 days) had no effect on recovery. The extraction technique (including the potassium chloride wash) has been accepted as giving quantitative extraction of lipids from soft tissues for many years[33]; this would permit expression of CDD/CDF levels relative to tissue lipid content, both determined in the same extract[31]. Evidence concerning extractability of CDDs and CDFs from exogenously fortified liver samples will be discussed later.

Soil. Gross characteristics of the types of soil used in this study, as of the time at which they were spiked with test compounds, are summarized in Table IV, as are the recoveries of ^{14}C-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from soil spiked at the 450-ptt (pg/g) level. This level was chosen because it is below the level considered significantly hazardous to human health[34], yet high enough to measure the radioactivity accurately.

TABLE III

EXTRACTABILITY OF ^{14}C-LABELED 2,3,7,8-TETRACHLORO DIBENZO-*p*-DIOXIN FROM RAT LIVER

^{14}C-labeled 2,3,7,8-CDD given orally in corn oil. Rats sacrificed after the number of days indicated; livers (1 g portions) extracted as described in the text.

Rat (strain)	*Time since dosing (days)*	*Tissue level (ng/g fresh)**	*Apparent percentage extracted****
101 (F344)	3	988 ± 24	99.7
102 (F344)	3	562 ± 14	99.4
103 (F344)	50	24.7 ± 0.6**	103.0
104 (F344)	70	10.3 ± 0.3	99.8
430 (CD)	3	20.2 ± 0.5	99.5
439 (CD)	3	17.7 ± 0.5	99.5

* Level of 2,3,7,8-CDD in the livers based on radioassay of tissue digests in NCS.

** Radioactivity in this liver measured using a tissue oxidizer. Digestion in NCS considered more reliable under these conditions, as it is not certain CDDs burn quantitatively to $^{14}CO_2$.

*** Based on the ratio of total ^{14}C in the chloroform phase after washing with aqueous potassium chloride (see text) to the mean total ^{14}C in the original tissue as determined by digestion. Extracts radioassayed with counting to a standard error of ± 1%. All extractable ^{14}C chromatographed with 2,3,7,8-CDD on a silica gel 60 thin-layer plate in hexane–methylene chloride (95:5)[30].

Extractability from the humic-rich loam was lower than that from other soil types ($p < 0.05$ *versus* sandy soil, 2-tailed *t*-test, $d_f = 4$), suggesting a strong interaction between the CDD and soil organic matter. However, extractability was sufficiently high and reproducible to be considered quantitative, even from loam. There was no difference between the extractabilities from kaolinite-based clay and bentonite (a montmorillonite-based clay used as an absorbant and having ion-exchange properties).

TABLE IV

EXTRACTABILITY OF ^{14}C-LABELED 2,3,7,8-CDD FROM SOIL OF VARIOUS TYPES

Portions of soil (20 g) were spiked with 9 ng of ^{14}C-labeled 2,3,7,8-CDD in acetone. The solvent was evaporated in a fume hood for 16 h prior to extraction as described in the text.

	Sandy soil (North Carolina)	*Red clay* (North Carolina)*	*Bentonite (Wyoming)*	*Loam (Indiana)*
Moisture content (%)	0.55	0.18	7.90	4.28
Loss on ignition (%)	3.64	7.30	11.63	14.14
Organic matter (%)	1.19	0.79	ND**	4.72
Extractable ^{14}C (%) (mean ± S.D., $N = 3$)	98.2 ± 1.81	99.7 ± 5.54	98.5 ± 1.71	93.7 ± 2.44
Recovered after cleanup, % of spiked***	96.2 ± 1.75	97.9 ± 5.42	97.1 ± 1.65	90.2 ± 2.22

* Primarily kaolinite.

** ND = None detectable (<0.2%).

*** In the final 20% methylene chloride fraction from acidic alumina. See text for details.

Final recoveries after two stages of cleanup (basic alumina followed by acidic alumina) are also summarized in Table IV (last row of numbers). The lowest mean recovery overall was 90.2%, for organic-rich loam. Almost quantitative recoveries (96.2–97.9%) were accomplished for the other soil types at this level of contamination (450 ppt).

Several samples of loam were spiked with 1.5 ppb (ng/g) of ^{14}C-labeled 2,3,7,8-CDD. Half the samples were extracted after standing for 16 h in a fume hood. The remaining samples were held in an open container, protected from light, for 30 days prior to extraction. The recovery from the "aged" loam was 94.4 ± 2.5% of that from the "non-aged" samples ($N1 = N2 = 3$), but the difference was not statistically significant ($0.2 > P > 0.05$). If there is increased tightness of binding with time, it was not conspicuous under these conditions. This will be more fully discussed later.

Considering organic-rich loam to be a "worst case" situation, the ^{14}C-labeled 2,3,7,8-CDD not recovered (Table IV) amounted to 7% of 450 ppt = 31.5 ppt. To determine whether this represented a low concentration of high affinity binding sites, a symmetry experiment was performed. Six 20-g portions of loam were processed in two groups of three samples each. One group was spiked as described with 50 ppt (pg/g) of non-radioactive 2,3,7,8-CDD and the other with 50 ppt of ^{14}C-labeled 2,3,7,8-CDD. Sixteen hours later the samples were spiked again; this time the former group received ^{14}C-labeled 2,3,7,8-CDD (50 ppt) and the latter group received 50 ppt of unlabeled 2,3,7,8-CDD. After the usual drying period, all samples were extracted as above except without the trichloro-CDD carrier, and the extracts were radioassayed.

The overall recovery of ^{14}C was 88.1 ± 1.7% ($N = 6$), which was not statistically significantly different from the recovery shown in Table IV for the 450-ppt level ($t = 0.4322$, $d_f = 6$, $P > 0.8$ of no significant difference). The unextracted portion here did not exceed 13 ppt and was as expected for a simple distribution coefficient between soil and extractant. There was no indication of the existence of saturable high affinity sites not freely exchangeable under these conditions.

Effectiveness of cleanup

Unspiked samples of rat liver (5 g), red clay (20 g) and loam (20 g) were extracted after addition of 10 ng of the trichlorodibenzo-*p*-dioxin carrier and 100 ppt of [^{13}C]tetrachlorodibenzo-*p*-dioxin internal standard. The extracts were processed through the chromatographic cleanup and examined by capillary column GC–MS, monitoring for the molecular ions at $m/z = 320$ and 322 for tetrachlorodibenzo-*p*-dioxins and at 332 for the internal standard, at a mass spectral resolving power of 10,000. The actual limit of detection was not determined on the day of this experiment, but 5 pg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin could easily be measured in the amount of sample injected. Total background noise in the channel monitoring the 322 ion in the scan region corresponding to the elution position of 2,3,7,8-CDD was corrected for the corresponding background in that region when only the ^{13}C-labeled 2,3,7,8-CDD was injected in benzene, and any remainder considered to represent potential interference not removed by the cleanup procedure. Fig. 1 illustrates the results observed when the extract of loam was analyzed. The random background noise in the 2,3,7,8-CDD region corresponded to a concentration of 0.3 ppt in the

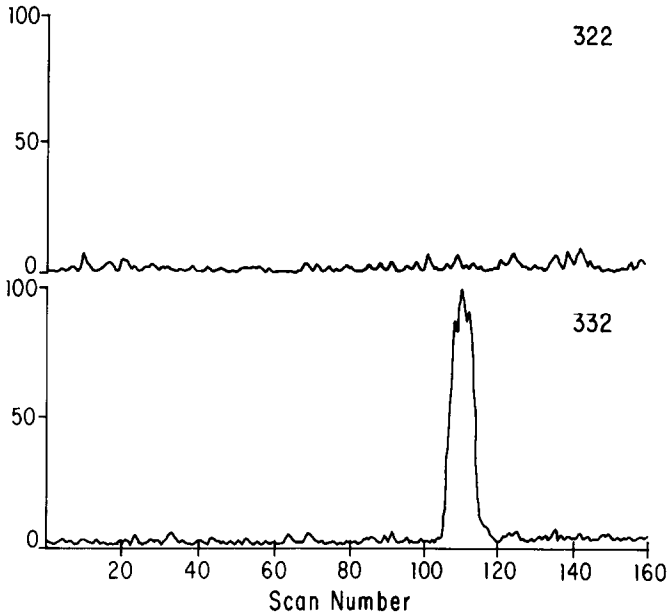

Fig. 1. Reconstructed single ion chromatograms from GC–MS of cleaned extract of humus-rich loam. Upper chromatogram, output of channel monitoring m/z = 322 for tetrachlorodibenzo-*p*-dioxins (all ^{12}C). Lower chromatogram, output of channel monitoring m/z = 332 for all ^{13}C-labeled 2,3,7,8-CDD which was added originally to the loam at the 100-ppt level. Background in upper channel between scans 103 and 120 (the 2,3,7,8-CDD region) equivalent to 0.3 pg of 2,3,7,8-CDD.

loam, and was not detectably higher than seen when an unprocessed blank solution of ^{13}C-labeled 2,3,7,8-CDD was analyzed. Thus the extract of loam soil was sufficiently freed of interferences to measure 2,3,7,8-CDD at levels at least down to 2.5 × 0.3 = 0.75 ppt (S/N = 2.5). This does not mean that such a low level of CDD would necessarily be recovered sufficiently for measurement, but that the freedom from interference would permit quantification at this level if recovery was adequate. Similarly, the cleaned extract of clay would have permitted measurement of 2,3,7,8-CDD down to a level of 2 ppt (S/N = 2.5), but liver could only be monitored down to 11 ppt under these conditions. Lower levels would not necessarily have been missed, but could not have been quantified accurately (S/N < 2.5). Tetrachlorodibenzo-*p*-dioxins eluting from the capillary column before the 2,3,7,8-isomer would have had a somewhat lower limit of detection and those eluting later a somewhat higher limit, relative to the background noise in different regions of the chromatogram. No "peaks" that might have been mistaken for tetrachloro-CDDs were observed in any of these runs.

Recovery through cleanup

We have found no detectable losses of CDDs or CDFs from monochloro- through octachloro-homologues regardless of sample load on LH-20 Sephadex chromatography, up to the limit of solubility of a given compound (lowest for octachloro-CDD) in methylene chloride–methanol (1:1, v/v). Up to 0.5 g of lipid (adipose

tissue extract or vegetable oil) can be run on 20 g of LH-20 without effect on the elution peak for 2,3,7,8-CDD. Mineral oil up to 0.5 g and total rat liver lipid up to 100 mg do not influence CDD-CDF elution positions. However, unchlorinated dibenzo-*p*-dioxin and dibenzofuran are only recovered to the extent of 95 ± 1.5% in the "aromatics" fraction, as they begin to elute slightly sooner than the chlorinated species. As indicated, the breakthrough volume for ^{14}C-labeled 2,3,7,8-CDD, for ^{3}H-labeled PCBs (similar to Aroclor 1248), or for anthracene (monitored by fluorescence) was independent of sample load between 1 ng and 1 μg of test compound. This confirmed that the chromatography involved linear distribution processes and was free of adsorption phenomena. Therefore the major consideration in this step was the degree of cleanup achieved.

Table II also summarizes the maximum percentage of carryover into the aromatics fraction of a variety of aliphatic materials applied to the LH-20 column. The loading was 0.5 ml for cottonseed oil and medicinal mineral oil, 100 mg for rat liver lipids (consisting mainly of phospholipids), and 25 mg for free fatty acids and cholesterol esters. Medicinal mineral oil consists primarily of condensed ring aliphatic hydrocarbons and is supposedly free of aromatics[35]. Elution was monitored on a weight basis, by determination of organic carbon as described by Amenta[36] or determination of total cholesterol as described previously[37].

From the results in Table II, one would expect at least 93% removal of aliphatic contaminants other than free fatty acids even at these high levels of loading. This is quite comparable to the results obtained by partitioning against concentrated sulfuric acid, but much more gentle and safe[30]. Since alumina can tolerate up to 20 mg of lipid per gram of adsorbant without overloading[30], this degree of preliminary cleanup should be quite adequate. In addition, and in contrast to the sulfuric acid procedure[30], the LH-20 column completely eliminates cholesterol esters, which otherwise would elute from subsequent alumina columns close to the CDDs + CDFs[30].

Chromatographic cleanup of CDDs and CDFs on these types of alumina columns has been discussed extensively in the literature (*e.g.* refs. 8, 38 and 39). Recoveries are essentially quantitative for CDD and CDF homologues other than the octachloro species, as far as elution from the columns are concerned. However, less than complete recoveries result from losses during evaporation of the solvents[40]. We processed cottonseed oil (0.5 g) spiked with either 10 or 100 ppt of 2,3,7,8-CDD through the entire cleanup procedure (LH-20, A-540 alumina, acidic alumina) with and without the trichlorodibenzo-*p*-dioxin carrier in duplicate, monitoring recovery by GC–MS. In this experiment the trichloro-CDD carrier level was only 20 ng per g lipid. Without the carrier the recovery, determined by adding isotopic internal standard just prior to analysis by GC–MS, was only 35 ± 5% at the 10-ppt level, and 49 ± 12% at the 100-ppt level, as would be expected from the observations of O'Keefe *et al.*[40]. In contrast, samples processed with the carrier gave mean recoveries of 87.7–97.9% (total range), or 92.8 ± 5.1%. Thus handling losses were almost eliminated through the use of a carrier that is slightly more volatile than the compounds of interest. The trichlorodibenzo-*p*-dioxin itself was analyzed and found to be >99.4% gas chromatographically pure, with no detectable (<0.04%) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin contamination.

Particular attention was paid to the ability of this cleanup procedure to separate polychlorinated biphenyls, polychlorinated diphenyl ethers and DDE from the

CDD + CDF fraction. There is, of course, no removal of these contaminants on LH-20.

The separation of ^{3}H-labeled PCBs (approx. 48% Cl) from ^{14}C-labeled CDFs is shown in Fig. 2, as is the separation of a large excess of unlabeled Aroclor 1254 from ^{14}C-labeled 2,3,7,8-CDD. In this case the column packing was A-540 alumina. The difference between the elution pattern for Aroclor 1254 on 3 g of A-540 alumina (2% methylene chloride in hexane) and on 3 g of acidic alumina (1% methylene chloride in hexane) is shown in the insert. Corn oil (50 mg) was included with each sample load; this amount of lipid is nearly but not quite sufficient to overload the column, and does not elute under the conditions shown. The load of CDD or CDFs was 20 ng, while the load of polychlorinated biphenyl was 150 μg. In other experiments the load of polychlorinated biphenyl mixture was varied from 37.5 μg to 1 mg.

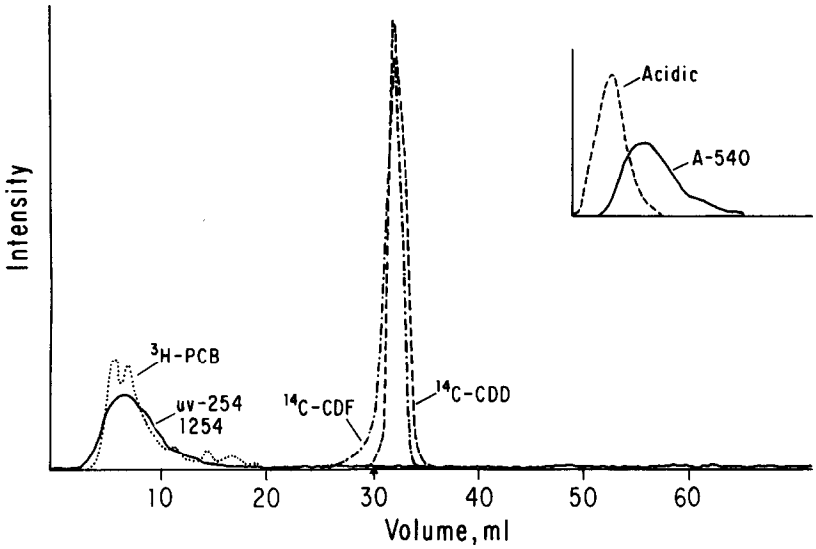

Fig. 2. Separation of polychlorinated biphenyls from CDD and CDF. Main figure: 3 g of A-540 alumina. Superimposed plots from two columns; separation of Aroclor 1254 (solid line, UV absorbance at 254 nm), 150 μg, from ^{14}C-labeled mixture of CDFs (10 ng) monitored by radioassay, dotted line. Also separation of ^{3}H-labeled PCBs (48% chlorine), dot-dash line, 1 μg, from ^{14}C-labeled 2,3,7,8-CDD (dashed line, 10 ng). Solvent, n-hexane–methylene chloride (98:2), changed to 80:20 at the arrow (30 ml). Insert, comparison of elution of Aroclor 1254 on 3 g of A-540 alumina with 2% methylene chloride in hexane, and on Merck acidic alumina with 1% methylene chloride in hexane.

For the chromatograms shown, the recoveries of ^{14}C-labeled 2,3,7,8-CDD and of ^{14}C-labeled CDFs in the 20% methylene chloride fractions were 99.2 and 93.4% of the load, respectively. It is not certain that all of the ^{14}C in the CDF preparation was in chlorinated material. In general, no less than 98% of the polychlorinated biphenyl mixture was eluted in the 2% methylene chloride fraction from A-540 alumina, and no less than 99% from the acidic alumina in 1% methylene chloride, up to a loading of 150 μg of total polychlorinated biphenyl. Beyond this point tailing became excessive and cleanup inadequate. The combination of columns, then, removes not less than 99.98% of the polychlorinated biphenyl contaminants from the

CDD + CDF fraction. Fig. 3 compares the elution patterns for polychlorinated biphenyls, naphthalenes (Halowax 1013) and diphenylethers (5–8 chlorines per molecule) from A-540 alumina. The naphthalenes and diphenylethers have less tendency to "tail" than the chlorinated biphenyls, and are therefore even more effectively eliminated in the cleanup. The pesticide DDE elutes, as far as could be determined, completely within 7 ml of 2% methylene chloride per g of A-540 alumina (not shown) and would not even reach the second column. It is necessary to point out, however, that the chlorinated methoxybiphenyls that occur in fish tissues[23] do come through this entire cleanup procedure in the CDD + CDF fraction. The same should apply to chlorinated benzyl phenyl ethers[22].

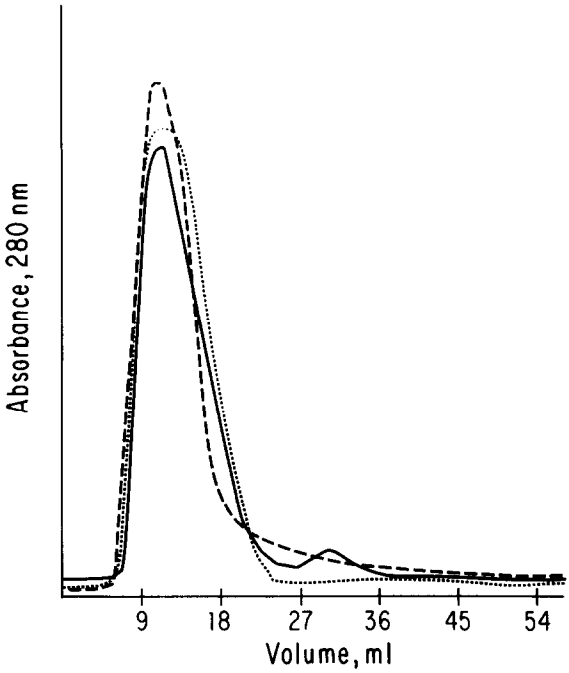

Fig. 3. Elution of Aroclor 1254 (PCBs, solid line), Halowax 1013 (chlorinated naphthalenes, dotted line) and polychlorinated (4–8 Cl) diphenyl ethers (dashed line) on 3 g of A-540 alumina. Load, 50 μg each mixture, monitored by absorbance at 280 nm. Superimposed plots from three columns, eluted with *n*-hexane–methylene chloride (98:2, v/v).

DISCUSSION

Extraction with chloroform–methanol (2:1), originally developed and extensively utilized for the recovery of total lipids from soft tissues[33], has been used in our laboratory for many years to release CDDs from liver for subsequent analysis by mass spectrometry[5] and radioimmunoassay[16]. Although adipose tissue is often preferred to liver for determination of residues of hydrophobic compounds, CDDs tend to accumulate in liver of rats[32,41], rabbits[42], chickens[7,42], hamsters[43] and cows[5] to a higher extent than in adipose. It has been reported that CDFs accumulate in rat

liver to a greater extent than do CDDs[44]. CDFs were readily determined in liver tissue samples from humans who had ingested contaminated rice oil[45]. Octachlorodibenzo-*p*-dioxin also reportedly accumulates in rat liver[46].

We have previously recommended partitioning against sulfuric acid for the reduction of lipids in extracts of CDDs[30]. This technique offers obvious safety hazards, and is ineffective at removing mineral oil or other saturated hydrocarbons. Chromatography on LH-20 Sephadex provides a similar degree of reduction in lipid content, while also removing aliphatic hydrocarbons. Use of LH-20 to generate a "total aromatics" fraction was described by Giger and Schaffner[47], who were concerned with the analysis of polycyclic aromatic hydrocarbons in soil. These authors used benzene–methanol (1:1, v/v), a solvent mixture we wished to avoid because of the toxicity of benzene. Dunn and Armour[48] substituted toluene–ethanol (1:1), with comparable results; however, we found this solvent mixture impossible to evaporate without a major loss of nanogram quantities of CDDs and CDFs. Methylene chloride–methanol (1:1), gave at least as good separations as benzene–methanol with much less toxicity and a much greater volatility.

The basis for the retardation of aromatic compounds on LH-20 Sephadex is thought to be charge-transfer interaction with the ether linkages in the gel[49]. Inclusion of methanol as a major component of the solvent prevents hydrogen bonding from retarding aliphatics[50], so they separate on a molecular size basis. Unfortunately, this allows the relatively low-molecular-weight free fatty acids to overlap into the aromatics fraction, but they are easily removed on basic alumina. Up to 50% by volume of non-polar solvent (benzene or methylene chloride) does not prevent the methanol from suppressing hydrogen bonding, but greatly increases the solubility of lipids and the non-polar pollutants in the solvent.

Previous studies involving liver samples fortified with a range of standard CDDs dissolved in DMSO[30] indicated that the recoveries of 2,3,7,8-, 1,2,3,6,7,8-, 1,2,3,4,6,7,8- and 1,2,3,4,6,7,8,9-CDDs were the same[5]. A slightly different cleanup procedure was used in the earlier studies[30], but the extraction method was the same as described here. Thus far no differences in the extractabilities of different CDDs has been seen using the chloroform–methanol procedure. The quantitative extraction of *endogenous* 2,3,7,8-CDD observed in this study implies that the final recovery of ^{13}C-labeled 2,3,7,8-CDD added as an exogenous internal standard, will indeed serve as a valid indicator of the final recovery (through cleanup) of endogenous 2,3,7,8-CDD and presumably other CDDs as well. The most widely used alternative extraction method for 2,3,7,8-CDD in tissues, which exists in a variety of modifications[51–53], involves alkaline hydrolysis of the tissue. This almost totally destroys the octachloro-CDD and -CDF[18,30], while producing lower chlorinated homologs as artifacts[18].

Methods for the recovery of CDDs from soil have with few exceptions been developed and validated exclusively relative to 2,3,7,8-CDD. These methods rely on either alkaline hydrolysis of the soil samples[54] or on extraction with a mixture of hexane and acetone containing either 20% acetone[55,56] or 50% acetone, with[20] or without[57] a pretreatment with aqueous ammonium chloride. Alkaline hydrolysis is unacceptable if the hexa- through octachloro CDDs or CDFs are to be measured, for the same reasons given above for liver. A method for determination of the series of CDDs from tetrachloro through octachloro in particulates has been described by Lamparski and Nestrick[58]. Unlike the methods intended solely for determination of

2,3,7,8-CDD mentioned above, the more versatile method involves Soxhlet extraction with refluxing benzene. Since this method is intended to be applicable also to fly ash from municipal incinerators, benzene (or toluene) is probably almost mandatory. Kooke *et al.*[59] have shown that the effectively encapsulated CDDs and CDFs in fly ash are extracted efficiently by the aromatic solvents, but other solvents (including hexane–acetone, 1:1) give very poor recoveries.

In every report from the conventional literature that has come to our attention, studies of the recovery of 2,3,7,8-CDD from spiked soil samples have involved the application of the CDD to the soil in a hydrocarbon solvent. (One exception to this generality, ref. 60, is not yet published at the time of this writing.) We believe this approach to be questionable, for the following reason. Soil particles always possess a surface coating of water adhering so tightly that it is effectively a solid[61]. The force of attraction between the soil particle and the water has been reported to be equivalent to a pressure of 10,000 atmospheres[61]. It is extremely unlikely that a hydrocarbon solvent could penetrate this "frozen" water layer, during a spiking procedure, in the time available prior to evaporation of the solvent. Thus the CDD "spike" will be left coated on the water layer, to which it may adsorb through hydrogen bonding only. Environmentally contaminated soil, which may have been contaminated a very long time prior to analysis, may well have slowly equilibrated to the point where the CDDs have managed to penetrate the water layer. If so, hydrophobic repulsion forces and charge-transfer interactions with constituents of clay or humic materials may be added to the interaction of hydrogen bonding and need to be overcome by the extracting solvent. The cohesive interactions contributing to the "frozen" nature of the water layer are considered to involve hydrogen bonding between water molecules[61]. For this reason the solvent in which CDD (and CDF) spikes, carriers, and internal standards are added to soil should preferably be capable of disrupting hydrogen bonds, and be miscible with water. We presently favor acetone for this purpose as do Bonaccorsi *et al.*[60], but tetrahydrofuran and other similar solvents should be equally effective.

Similarly, diluting acetone with hexane as an extractant can only slow its disruption of the (potentially) protective water layer. By effectively "soaking" the soil with acetone prior to elutriation with a less polar solvent, we minimize the time and the amount of solvent needed to expose and extract the pollutants. We prefer ethyl acetate to hexane simply because only a moderate amount of water in the soil will cause an acetone–hexane mixture to split into two phases, while acetone–ethyl acetate is more tolerant. We complete the extraction with methylene chloride primarily in order to raise the vapor pressure of the solvent mixture, simplifying the evaporation step and minimizing losses. Methylene chloride is not applied while the acetone is still present, as the heat generated by their mixing would tend to cause channeling and bubble formation.

Extraction of soil with hexane:acetone requires at least three[20] and in some cases six[56] repetitive applications of solvent. Soxhlet extraction is an inherently slow process, and permits only one sample to be processed per set of moderately elaborate apparatus. The present method requires no apparatus more elaborate than a separatory funnel, any number of samples can be extracted simultaneously, and a relatively small volume of solvent (164 ml total for 20 g of soil) is required. This procedure is not adequate for fly ash, and, since aromatic solvents are not used, probably not

suitable for samples containing soot or charcoal[25]. However, it is clearly effective for a variety of types of natural soil.

We have not determined the recoveries of CDDs and CDFs from liver or soil at levels of content below *e.g.* 100 ppt in this study. In regard to liver, we are unable to use radiolabeled test compounds to measure independently levels of endogenous pollutants below about 1 ppb (ng/g), and we would prefer not to take it for granted that recovery of exogenous spikes necessarily would correspond to recovery of endogenous material[29], with no way to confirm that supposition. This objection does not apply to soil, since there is no clear conceptual distinction between endogenous and exogenous in that case. Nonetheless, we would not trust a value for recovery at low ppt levels determined for one type of soil and applied to another. Thus recovery studies at extremely low levels should be applied to the specific matrix of concern in a given study.

The present study indicated that the extraction and cleanup procedure here described, when applied to an uncontaminated matrix, gave a product sufficiently clean to permit low ppt measurements for 2,3,7,8-CDD (in the sense of freedom from false positives), and that it gave very high recoveries at the 450-ppt level. The special advantages of this approach are the very gentle conditions employed, the avoidance of hazardous reagents, the absence of any indication of differential recoveries within a class (except for slight losses of the octachloro species) and the relative speed of the process. Two technicians can fully process about 24 samples per five-day week if necessary. This may be compared to a recently published, semi-automated high-performance liquid chromatography procedure for 2,3,7,8-CDD in fish which requires five days per technician per nine samples, yet was a significant improvement in speed over previous methods[62].

The disadvantages of the present approach derive primarily from the fact that it is intended to permit recovery of Cl_4–Cl_8 CDDs and CDFs in general and not any one in particular. Thus the cleanup procedure stops short of the enrichment probably needed for analysis at the 1-ppt level, in order to avoid selective losses of some of the CDD and CDF isomers. If the objective of a study is, for example, measurement of 2,3,7,8-CDD or -CDF at lowest possible detection limits, an approach other than the one described here should be chosen.

A second possible disadvantage of the present approach is a general problem common to methods involving chromatography on alumina. The occurrence of extremely high levels of PCBs in extracts can result in an ill-defined interaction with CDFs, causing them to elute from alumina even with 2% methylene chloride in hexane. Thus, since one of the commonest sources of CDF contamination is their occurrence as impurities in commercial PCBs, and since the approximate maximum load of PCBs that will not result in premature elution of CDFs is 50 μg per g of A-540 alumina (unpublished observations), the original sample matrix (*e.g.* soil) should not contain more than 7.5 ppm (μg/g) of polychlorinated biphenyls if the recovery of CDFs implied in this paper is to be possible.

The general approach to sample workup described here has been successfully applied in bioavailability studies involving contaminated soil from Times Beach, Missouri[63] and, with the exception of omission of the LH-20 column, to analysis of CDDs in beef liver[5]. We have, however, experienced difficulties in obtaining reproducible recoveries from adipose tissue, which is still under study.

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