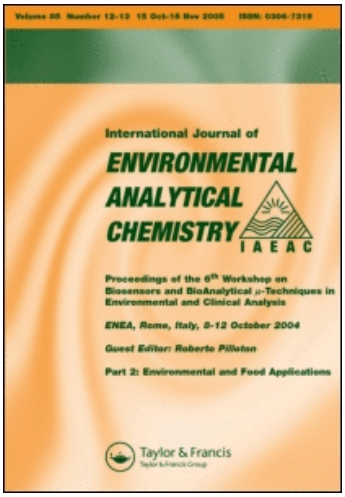

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A Gel-Permeation/Column Chromatography Cleanup Method for the Determination of CDDs in Animal Tissue

R. J. Norstrom[a]; M. Simon[a]; M. J. Mulvihill[a]
[a] Environment Canada, Canadian Wildlife Service National Wildlife Research Centre, Ottawa, Canada

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A Gel-Permeation/Column Chromatography Cleanup Method for the Determination of CDDs in Animal Tissue

R. J. NORSTROM, M. SIMON and M. J. MULVIHILL

Environment Canada, Canadian Wildlife Service
National Wildlife Research Centre, Ottawa, Canada KIA 0E7

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A method which can be implemented in a routine organochlorine analysis laboratory equipped with a quadrupole GC/MS is presented for determination of tetrachloro- (T_4-) to octachloro- (O_8-) dibenzodioxins (CDDs) in animal tissues. Long chain-length biogenic compounds such as lipids are removed by gel-permeation chromatography (GPC), and the CDDs are separated from most interfering organochlorine compounds by a combination of alumina and Florisil chromatography. Recoveries from spiked samples were in the 87–97% range for T_4CDDs–H_7CDDs at the 5–100 ng/kg level except for 1,3,6,8- and 1,3,7,9,-T_4CDD, which had variable losses in the alumina chromatography step (recoveries of 73–97%). O_8CDD recoveries depended on level, 80% at 200 ng/kg, 52–59% at 30–40 ng/kg. Precision of replicate analysis was generally in the 2–7% range. Positive interferences not completely removed by the method were identified as methoxychloro-biphenyls and methoxychloro-diphenylethers. Examples of the application of the method are given.

KEY WORDS: PCDD, dioxin, determination, tissues.

INTRODUCTION

As discussed by Crummett,[1] appropriate combinations of "resolving power" in sample preparation, gas chromatography and mass spectrometry can be used to accomplish a high degree of specificity for ultra-trace (ng/kg) determination of chlorinated dibenzodioxins (CDDs). The analytes may be efficiently separated from the matrix and interfering xenobiotics ("cleanup") so that relatively sensitive and non-specific detection methods such as electron-capture gas chromatography (EC/GC) may be used, or a less specific cleanup may be combined with sensitive and highly specific detection methods such as high resolution gas chromatography/high resolution mass spectrometry or mass spectrometry/mass spectrometry.

A comparison of methodology has been reported for 2,3,7,8-TCDD in fish tissue.[2] It is clear from these studies that the specificity required for EC/GC determination is not easily attained. For the routine laboratory faced with determination of CDDs, and possessing only a quadrupole mass spectrometer, specific cleanup techniques must therefore be employed. The first problem facing the chemist in analysis of biological samples is removal of the lipids co-extracted with the CDDs. Saponification of the sample is often used to destroy lipids prior to extraction,[3] but some higher chlorinated CDDs are labile under these conditions. Neutral extraction followed by destruction of lipids with concentrated sulfuric acid is also used.[4,5] However, this procedure removes oxygenated organochlorines such as oxychlordane and dieldrin which we wished to determine. Our original method[6] therefore used acetonitrile/hexane partitioning for bulk lipid removal. This step was satisfactory in T_4CDD determination, but gave very low recoveries for O_8CDD.[7] The method was therefore modified to replace the partitioning step, along with other refinements.

We wish to report an improved method for cleanup and determination of T_4CDD–O_8CDD in biological samples which uses the automated gel-permeation chromatography (GPC) procedure of Stalling *et al.*[8] for removal of lipids, and simple column chromatography steps for separation of CDDs from remaining co-extractives and most other organochlorine compounds. The method can be easily implemented in a normally-equipped residue analysis laboratory.

MATERIALS AND METHODS

Materials

Solvents Pesticide analysis grade *n*-hexane, dichloromethane (DCM) and toluene.

Sodium sulfate Granular anhydrous. Heat in 400° oven overnight, wash with hexane, air dry and store in a tightly-closed container.

Bio-beads S-X3 Bio-Rad Laboratories, 200–400 mesh. Swell 60 g in 50% (v:v) DCM:hexane overnight, and pack the slurry into an Analytical Biochemistry Laboratories 2.5 cm i.d. GPC column. Connect the column to an Analytical Biochemistry Laboratories Auto-Prep 1002 Gel Permeation Chromatograph (upward flow) and begin pumping at 5 ml/min. Compress the bed length to approximately 43 cm. If the bed tends to rise from the bottom when under pressure, adjust the bottom plunger to eliminate dead volume.

Alumina (large column) Fisher A950, neutral, Brockman activity 1, 80–200 mesh. Activate at 550° for 6 hr, deactivate 1% (w:w) with distilled, hexane extracted water and store in a tightly closed container. Pack a 2 cm i.d. × 35 cm glass column (Teflon stopcock) with a glass wool plug and 30 g alumina just prior to use.

Florisil PR grade, Floridin Corporation. Activate at 600° for 6 hr, deactivate 1.2% (w:w) with distilled, hexane extracted water and store in a tightly-closed container. Pack a 1 cm i.d. × 50 cm glass column (Teflon stopcock) with a glass wool plug, 17 g Florisil and 2 cm sodium sulfate just prior to use.

Alumina (microcolumn) Fisher A948, acid, Brockman activity 1, 60–200 mesh. Activate at 550° for 6 hr. Pack Pasteur pipettes with glass wool plug, 1 g alumina and 5 mm sodium sulfate. Store the columns at 190° until needed.

Primary internal standard ($^{13}C_{12}$)-2,3,7,8-T_4CDD and ($^{13}C_{12}$)-O_8CDD, KOR Isotopes. Make a mixture containing 50 ng/ml each of T_4CDD and 100 ng/ml of O_8CDD in toluene and add 25 μl to a 25 g sample (concentration of 50 pg/μl T_4CDD and 100 pg/μl O_8CDD if final volume is reduced to 25 μl). Prepare frequently from stock solution.

Secondary internal standard ($^{37}Cl_4$)-2,3,7,8-T_4CDD, KOR Isotopes. Make a solution of 50 ng/ml in toluene and add 25 μl to a 25 g sample just prior to final volume adjustment.

External CDD standards 2,3,7,8-T_4CDD, 1,2,3,7,8-P_5CDD, 1,2,3,6,7,8-H_6CDD, 1,2,3,4,6,7,8-H_7CDD and O_8CDD, KOR Isotopes. These isomers are the ones most frequently found in biota. Make a mixed standard in toluene at the 50 ng/ml level for 2,3,7,8-T_4CDD, 100 ng/ml for all others, and add all labelled CDDs at the same level as the primary internal standard.

Sample preparation and extraction

Homogenize tissue to a paste consistency using a blender, polytron or other suitable apparatus, weigh out 25 g into a tared glass mortar, add 25 μl primary internal standard solution to the centre of the homogenate with a microsyringe and let stand 0.5 hr. The spiking level is 50 ng/kg for T_4CDD and 100 ng/kg for O_8CDD. Grind the sample thoroughly with 125–150 g anhydrous sodium sulfate, pour the mixture into the extraction column (3 cm i.d. × 55 cm glass with teflon stopcock), rinse out the mortar and pestle with portions of the extracting solvent (500 ml hexane) and extract the column with the remaining solvent at about 5 ml/min into a 1 l evaporating flask. Evaporate extract to approximately 1–2 ml with a rotary evaporator. Transfer to scintillation or other shallow vial with 50% (v:v) DCM:hexane and make up to 10 ml. The lipid concentration should not be more than 0.25 g/ml for the GPC separation, so this final volume is suitable for most tissues with relatively low lipid content ($<10\%$). If the sample has a high lipid content, a larger final extract volume and more loops on the GPC will be required.

Gel permeation chromatography

Use an Analytical Biochemistry Laboratories Autoprep 1002 chromatograph. This instrument is equipped with 23 × 5 ml injection loops and has timer controlled dump/collect/wash cycles. Attach a fluorocarbon Luer adapter with a Mininert valve to the inlet tube. For a 10 ml extract containing <2.5 g lipid, transfer the extract quantitatively to three injection loops of the GPC chromatograph as

follows. Aspirate the extract completely into a 10 ml Gastight syringe (Hamilton) equipped with a removable needle, up-end the syringe, remove the needle, push air out of the syringe, and attach a stainless steel Swinny filter (13 mm, Millipore) containing only the filter support screen to remove any larger particles that may be in the sample. Index the GPC to the correct loop, set the GPC to the "load" position, attach the syringe and filter to the Luer-lock/Mininert valve on the GPC inlet and open the Mininert valve. Inject 5.5 ml (i.e., about 1 ml less than the volume of the loop plus the dead volume in the connections) into the first loop, index to the second loop and inject the remaining contents of the syringe. Rinse out the vial, needle, syringe, and filter 3–5 × with approximately 1 ml volumes of 1:1 DCM:hexane, and inject into the third loop. The dead volume before the loops is approximately 1.5 ml, therefore the total volume injected into the last loop must be sufficient to flush this volume, but should not exceed 6 ml for quantitative transfer.

As many samples can be run automatically as there are loops to accommodate them. Place all the effluent tubes for the loops belonging to each sample in an appropriately sized evaporating flask. If desired, total lipid can be determined gravimetrically by collecting and evaporating the dump fractions from each sample.

A 50% DCM:hexane solvent system was chosen because we obtained better separation between lipids and a wide range of organochlorine compounds than with 50% DCM:cyclohexane as used by Stalling *et al.*[8] Hexane is also less expensive than cyclohexane and is somewhat more volatile. Elution profiles of several dioxin isomers spiked to 25 g of chicken egg were determined to establish the discard/collect/wash time sequence, as shown in Figure 1. The dumped fraction was set at 150 ml and the collected fraction at 130 ml. Approximately 99% of the lipid and about half of the carotenoid pigments were removed in this sequence. The collected fraction also contained the common organochlorine compounds such as PCBs, DDE, dieldrin and mirex.

Alumina chromatography cleanup

Evaporate the combined GPC eluates for each sample to 1–2 ml with a rotary evaporator, add 10 ml of hexane and reduce again to 1–2 ml. Repeat this step once to ensure elimination of DCM.

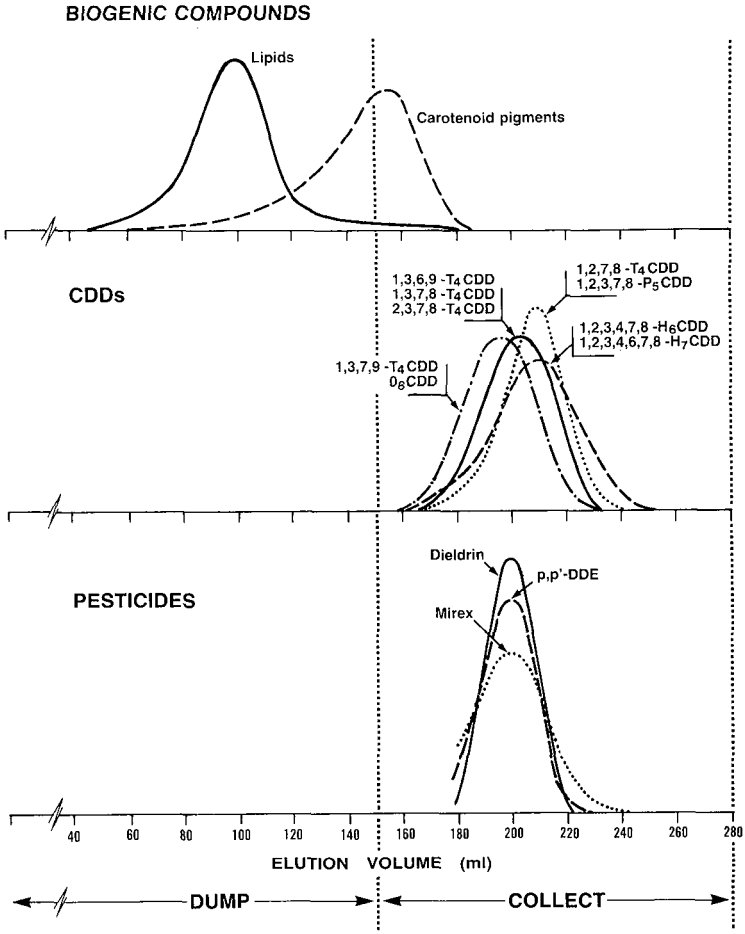

FIGURE 1 Elution profile of T_4CCDs to O_8CCDs, organochlorine compounds, chicken egg yolk lipids and carotenoid pigments from a 60 g Biobeads SX-3 column: 1:1 (v:v) DCM:hexane mobile phase. See text for experimental details. Lipids were determined gravimetrically, and carotenoids by visible light colorimetry.

Transfer to the alumina column with small portions of hexane. Elute the column with 150 ml 1% (v:v) DCM:hexane (Fraction 1) and collect for determination of PCBs, DDE, mirex and other non-polar organochlorine compounds if desired. Elute the CDDs with 150 ml 50% (v:v) DCM:hexane into an evaporation flask.

After this step, the sample still contains DDT, DDD, hexachlorocyclohexane, isomers, chlorinated naphthalenes, chlordane derivatives, heptachlor epoxide, dieldrin, and minor amounts of PCBs, but is relatively free of biogenic compounds. Attempts to adjust the first fraction volume to eliminate organochlorine compounds resulted in some loss of 1,3,6,8- and 1,3,7,9-T_4CDD. A Florisil column separation step was therefore introduced, because preliminary experiments showed that Florisil, as compared to alumina, retained CDDs more strongly than other organochlorines.

Florisil chromatography separation

Evaporate the second fraction from the alumina column in the same fashion as the GPC eluate, and transfer to the Florisil column with a small volume of hexane. Elute the column as follows: 100 ml hexane, 100 ml 5% (v:v) DCM:hexane, 100 ml 25% DCM:hexane, 100 ml DCM. The first two fractions contain most of the remaining interfering organochlorine compounds, and are discarded or analyzed for these compounds if desired. The 25% DCM:hexane fraction contains 94% of the 2,3,7,8-T_4CDD along with dieldrin and some heptachlor epoxide. The latter two compounds are well separated from TCCDs by capillary column GC, and do not pose any difficulties in the analysis. This fraction can be analyzed separately to minimize interferences if only T_4CDD is being determined. The last fraction contains all of the O_8CDD and the remainder of the 2,3,7,8-T_4CDD. Note that if the Florisil is not deactivated with water, O_8CDD is very difficult to elute. Because interferences were minimal in the last fraction of the samples we investigated, the last two fractions were combined. If preliminary GC/MS analysis indicates PCB interferences remaining at m/z 324, proceed to the alumina microcolumn chromatography step; otherwise proceed to final volume adjustment and secondary internal standard addition.

Alumina microcolumn chromatography

Interfering peaks at m/z 324 from pentachloro-PCBs often remained after the preceeding steps in samples containing more than 30 mg/kg of PCBs. To minimize this interference, chromatograph the combined fractions 3 and 4 from the Florisil column on a 1 g alumina

microcolumn. Evaporate the combined fractions to near dryness with a rotary evaporator, dissolve in 0.5–1.0 ml hexane, transfer to the column, elute with 8 ml 1% (v:v) DCM:hexane (discard) and then with 8 ml 50% (v:v) DCM:hexane, which is collected in a 15 ml centrifuge tube. The second fraction contains T_4CDD-O_8CDD.

Preparation for GC/MS Determination

Evaporate combined fractions 3 and 4 from the Florisil column to near dryness with a rotary evaporator (or fraction 2 from the alumina microcolumn with a gentle stream of purified air or nitrogen) and transfer quantitatively to a 100 μl Reacti-vial with small portions of toluene. To contain the rinsings, evaporate the toluene with a gentle stream of purified air or nitrogen between additions. Store the sample upright and in the dark in 100 μl of toluene.

Just prior to analysis, spike the sample at the 50 ng/kg level with secondary internal standard and evaporate to approximately 25 μl (1 g/μl). The m/z 328 ion from this standard is monitored in samples and external standards to compensate for changes in final sample volume and GC/MS sensitivity.

Capillary column GC/low resolution MS is used for quantitation. Our laboratory uses a Hewlett–Packard 5987B GC/MS. The electron ionization mode at 70 eV is employed because sensitivity of T_4CDDs is higher, and the response factor of the molecular ion of CDDs is probably more independent of chlorine placement on the ring than in chemical ionization.[9] It is therefore possible to quantitate approximately those CDDs for which no standards are available, provided that at least one isomer of each congener group is present in the standard.

Routine GC/MS analysis is performed on a 30 m DB-5 column because the high maximum operating temperature allows O_8CDD to be eluted in a reasonable time with good peak shape (Figure 2). A limited number of isomers are usually found in biota, and this column therefore provides sufficient resolution for isomer specific analysis. Unambiguous assignment of peak identities, however, requires injection of authentic standards. Route the column directly into the ion source to minimize possible losses of higher chlorinated dioxins. The GC oven temperature program is as follows: hold at

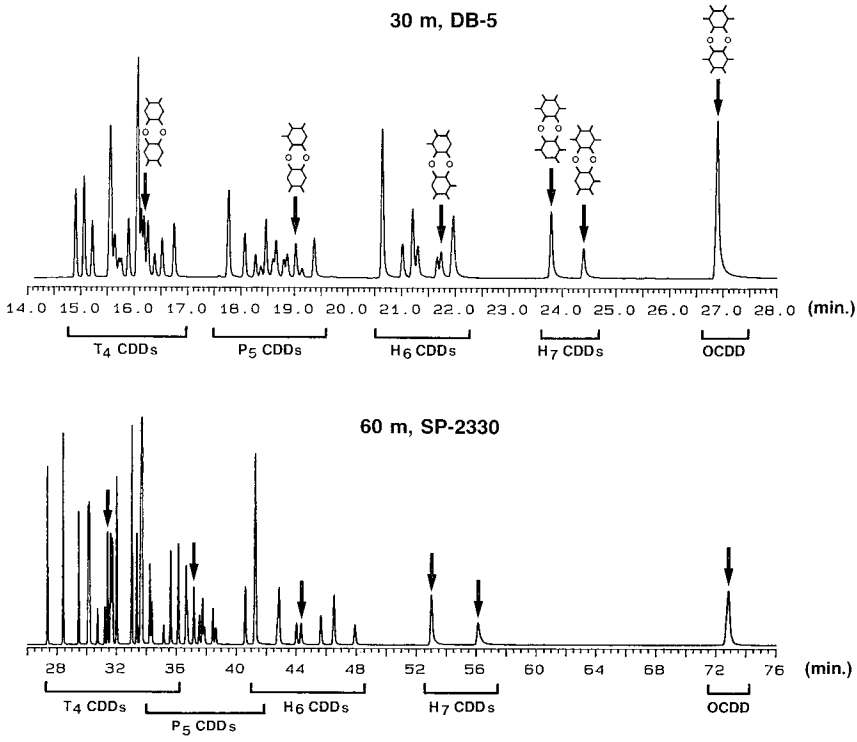

FIGURE 2 Chromatograms of all CDD congeners on a 30 m DB-5 thin-phase fused silica column (J & W Scientific), and a 60 m SP2330 fused silica column (Supelco, Inc.). The principal congeners found to date in animal tissues are indicated by the arrows. They are: 2,3,7,8-T_4CDD, 1,2,3,7,8-P_5CDD, 1,2,3,6,7,8-H_6CDD, both H_7CDD isomers and O_8CDD. These congeners are uniquely separated on both columns except 2,3,7,8-T_4CDD, which is separated only on the SP-2330 column.

100° for 3 min, 100°–180° at 20°/min, then 180°–230° at 5°/min. Samples may be injected using a splitless or on-column injector. In our experience, there is some sensitivity gain from on-column injection, particularly for O_8CDD, but this is more than offset by the smaller volumes (ca. 1 μl) which must be injected for good chromatography when compared to splitless injection, which can easily handle 3 μl.

Screening analysis

To screen for the presence of CDDs, determine the retention time window for each congener group (there are no overlaps on a DB-5 column between groups for the CDDs, see Figure 2), and set up the mass spectrometer in SIM mode to scan the three most intense ions in the molecular ion cluster and the $(M–COCl)^+$ diagnostic ion for each window. A dwell time of 50 ms for each ion is a good compromise between signal/noise and peak definition requirements under normal chromatographic conditions. Inject a 3 μl sample. Measure the areas at all m/z for candidate CDD peaks in each window.

The ion intensity ratios in the molecular ion cluster should be within $\pm 10\%$ of those determined from analysis of an authentic standard under the same GC/MS conditions in order for identification of a CDD to be made. An $(M–COCl)^+$ ion should always be present, but the relative abundance of this fragment varies with chlorine placement. In the case of 2,3,7,8-T_4CDD, it is 30–40%. Correct molecular ion cluster ratios and absence of $(M–COCl)^+$ can signal the presence of methoxy-PCBs (MeO–PCBs). See sections below on Interferences and Application of the Method.

Isomer specific identification of CDDs is relatively simple in most animal tissues, because only those isomers with 2,3,7,8- chlorine substitution are usually present.[10–12] The order of elution of all CDDs on a highly polar Silar 10-C capillary column has been determined by Buser and Rappe.[13] Silar 10-C has similar separation properties to SP2330, and a 60 m SP2330 column (Supelco Inc.) completely resolves 2,3,7,8-T_4CDD from other T_4CDD isomers (Figure 2). We therefore use this column for identification purposes. It is not used for routine analysis because of an overlap between some congener groups (especially T_4CDDs and P_5CDDs), and because O_8CDD elutes at a temperature near the upper limit of the column. Install the column in the ion source and analyze the sample as before, except that the second ramp in the temperature program should be at 3°/min to a maximum of 250°. If the correct molecular ion cluster ratios and $(M–COCl)^+$ are obtained as before, and there is no ambiguity in cross-matching peaks between the DB-5 and SP2330 columns, the isomer identity can be established if authentic standards are available to the analyst.

Quantitative analysis

To quantitate, install the DB-5 column and set up the GC/MS to monitor the strongest ion for the primary internal standard (m/z 334 or 472 in our case), the strongest ion for the secondary internal standard (m/z 328) and the strongest ion in the molecular ion cluster for each window. The dwell time for all ions is 100 ms. Inject a 3 μl sample and measure the areas. If a peak is near the instrumental detection limit, measure the peak height to noise ratio. Only peaks with a ratio >3 are considered to be above the minimum detectable amount (MDA).

We have found that the accuracy of ($^{13}C_{12}$) internal standards is not always reliable, based on comparison of response factors with unlabelled standards. To overcome this problem, we analyze an external standard mixture containing unlabelled CDDs as well as the primary and secondary internal standards. The external standard mixture is analyzed at least once each day under the same conditions as the samples. The recoveries of ($^{13}C_{12}$)-T_4CDD and ($^{13}C_{12}$)-O_8CDD are calculated by comparing relative areas of the peaks for these and ($^{37}Cl_4$)-T_4CDD in the sample and external standard chromatograms. These recoveries serve as a general indication of the quality of each determination. Levels of P_5CDDs to H_7CDDs for which no isotopically labelled internal standards are present can be corrected for recovery using the ($^{13}C_{12}$)-T_4CDD recovery, which is similar (see Recovery Studies below), or corrected only for volume and GC/MS sensitivity changes using the secondary internal standard. The calculations for both methods are illustrated below:

$$[\mathrm{CDD}_x],\ \mathrm{ng/kg} = (L_x A_x \underline{A}_s)/(A_s \underline{A}_x) \qquad (1)$$

where:

L_x = equivalent level of CDD_x in external standard relative to ($^{13}C_{12}$)-T_4CDD internal standard; i.e., 50 ng/kg for 2,3,7,8-T_4CDD, 100 ng/kg for all other CDDs.
A_x = area of CDD_x in sample chromatogram.
$\underline{A}_s$ = area of ($^{13}C_{12}$)- or ($^{37}Cl_4$)-T_4CDD in standard chromatogram.
A_s = area of ($^{13}C_{12}$)- or ($^{37}Cl_4$)-T_4CDD in sample chromatogram.
$\underline{A}_x$ = area of CDD_x in standard chromatogram.

Levels of O_8CDD are calculated using the ($^{13}C_{12}$)-O_8CDD internal standard.

Quality control protocol

Every sample is spiked with a ($^{13}C_{12}$) internal standard mixture at the time of extraction. This has been shown to be the most important factor in improving accuracy in the analysis of 2,3,7,8-T_4CDD in fish.[14] Recently ($^{13}C_{12}$) labelled P_5CDDs, P_6CDDs and P_7CDDs have become commercially available (Cambridge Isotopes), and these will be included in future internal standard mixtures. Every sixth sample is an uncontaminated or contaminated quality control sample of similar nature to the samples being analyzed. We have found that lipid collected from the dump cycle of the GPC makes an ideal uncontaminated sample if no other is available, because the CDDs and other OC contaminants have been stripped away. A pool of contaminated control samples can be prepared by thoroughly homogenizing together portions of the samples to be analyzed. Analysis of the quality control samples provides a measure of the precision of determination, and ensures that no external or cross-contamination of samples is occurring.

METHOD VALIDATION

Recovery study

The method was evaluated by analyzing egg homogenate spiked with a mixture of 12 CDDs at two different levels. One level was well above the minimum detectable amount (50–250 ng/kg), and the other was near this minimum (5–30 ng/kg). Herring gull eggs from an Atlantic Ocean colony were used because they contained <5 ng/kg 2,3,7,8-T_4CDD and no detectable levels of other CDDs. The eggs were homogenized, spiked prior to extraction in the same way as the internal standard, and carried through the whole procedure. At least four replicate analyses were performed. The results are given in Table I.

At the high spiking level, recoveries were 90% better for all compounds except 1,3,6,8-T_4CDD and O_8CDD, which were in the 75–80% range. The CV at the high spiking level ranged from 2–8% for all CDDs except 1,3,7,9-T_4CDD, which is excellent precision for ultra-trace analysis. The low spiking level was adjusted to be approximately three times the minimum detectable amount (MDA in Table I). Recovery and precision remained excellent at this low level

TABLE I

Recovery of CDDs from herring gull eggs spiked at two levels

CDD isomer	High level (ng/kg)	N	Recovery (%) Mean	(SD)	Low level (ng/kg)	N	Recovery (%) Mean	(SD)	MDA[a] (ng/kg)
1,3,6,8-T_4	100	4	78	(4)	10	4	89	(7)	3
1,3,7,9-	50	4	97	(15)	5	4	73	(5)	3
1,3,7,8-	100	4	90	(9)	10	4	95	(4)	3
2,3,7,8-	50	4	98	(3)	10	7	97	(4)	3
1,2,7,8-	100	4	91	(5)	10	7	89	(4)	3
1,2,3,7,8-P_5	100	4	96	(2)	20	7	92	(7)	6
1,2,3,4,7,8-H_6	100	4	105	(6)	20	7	87	(5)	6
1,2,3,4,6,7,9-H_7	100	4	102	(5)	—	–	—	—	—
1,2,3,4,6,7,8-	100	4	100	(3)	30	6	92	(7)	8
O_8	250	4	80	(8)	30	7	59	(17)	10
($^{13}C_{12}$) internal standards:									
2,3,7,8-T_4	200	13	98	(8)	10	7	98	(7)	3
O_8	200	13	80	(13)	40	7	52	(13)	10

[a]MDA = minimum detectable amount based on 3 × signal-to-noise.

for all CDDs except O_8CDD, which had relatively poor recovery (59%) and precision (CV = 17%). However, the ($^{13}C_{12}$)-O_8CDD recovery and precision paralleled that of the unlabelled O_8CDD, indicating that the internal standard compensated for low and variable recoveries.

Losses of O_8CDD probably occur because of incomplete transfer from evaporating flasks between the GPC/alumina, alumina/Florisil and Florisil/micro-alumina steps. In all cases the final solvent is hexane, in which O_8CDD is poorly soluble. We experimented taking a sample to dryness in a clean Reacti-vial and found that only 66% of the O_8CDD could be redissolved in hexane after several hours of sitting, the rest remained adsorbed to the glass surface. It is possible that addition of small quantities of toluene during transferring would improve the recovery of O_8CDD without affecting the subsequent steps.

Losses of 1,3,6,8- and 1,3,7,9-TCDD occur primarily in the first alumina chromatography step. Separate recovery experiments showed up to 15% of these isomers in the first fraction. If desired, the volume of the first fraction can be reduced to obtain better recovery of these isomers at the expense of having more PCBs present in the dioxin fraction. The subsequent Florisil chromatography step will remove most of this PCB contamination.

Note that recovery data at high levels of ($^{13}C_{12}$) internal standards in Table I were obtained from routine analysis of a wide variety of sample types, including liver, muscle, fat and eggs. These data clearly show the suitability of the method for analysis of animal tissues generally.

Interference mixture study

Smith and Johnson[15] tested their CDD and CDF analysis method for interference from a mixture of chlorinated compounds that have major ions at various m/z utilized for CDD and CDF analysis. Many of these compounds are well-known environmental contaminants, such as polychlorinated naphthalenes (PCNs), polychlorinated diphenyl ethers (PCDPEs) and PCBs, or are metabolites of these compounds, such as methoxy-PCBs (MeO–PCBs). We tested our method with the same mixture of compounds spiked at the 4 μg/kg level to 25 g of chicken egg. The results are given in Table II.

TABLE II

Removal of Inference Mixture compounds (Smith and Johnson[15]) from chicken eggs by alumina and Florisil chromatography

Compound	Percent removed
Polychlorinated biphenyls (PCBs)	
3,4,3′,4′-tetrachloro-	56
3,4,5,3′,4′-pentachloro-	64
3,4,5,3′,4′,5′-hexachloro-	76
Polychlorinated naphthalenes (PCNs)	
unidentified hexachloro-	100
Chloro diphenyl ethers (CDPEs)	
3,4,3′,4′-tetrachloro-	62
2,4,5,2′,4′-pentachloro-	82
2,3,4,5,2′,3′,4′-heptachloro-	71
2,3,4,5,6,2′,3′,4′-octachloro-	100
Decachloro-	100
Hydroxychloro biphenyls (HO–PCBs)	
2,3,4,5,3′-pentachloro-2-hydroxy-	100
2,3,5,2′,3′-pentachloro-2-hydroxy-	100
Methoxychloro diphenyl ethers (MeO–CDPEs)	
3,4,5,2′,4′-pentachloro-2-methoxy-	84
Nonachloro-2-methoxy-	100
Nonachloro-3-methoxy-	100
Methoxychloro biphenyls (MeO–PCBs)	
3,4,3′,4′-tetrachloro-2-methoxy-	68
2,4,3′,4′-tetrachloro-3-methoxy-	71
3,5,3′,4′-tetrachloro-4-methoxy-	67

Seven of the seventeen compounds were removed completely by the cleanup, and the remainder were at least 50% removed. The most serious potential interferences appear to be non-*o,o′* substituted PCBs (56–76% removed) and MeO–PCBs (67–71% removed). In both cases, the criteria for identification of CDDs given in the screening analysis above are sufficient to rule out false positive interference. Interference is only present at the third and higher ions in the molecular ion cluster of the CDD with one fewer chlorine in the case of PCBs (pentachloro-PCBs occur in the same chromatographic window as T_4CDDs), and no $(M–COCl)^+$ is present. In the

case of MeO–PCBs the interference is more serious, since the nominal masses in the molecular ion cluster have the same pattern as the corresponding CDD, and these compounds occur in the same chromatographic window as T_4CDDs. However, absence of the CDD diagnostic ion $(M–COCl)^+$ and presence of the MeO–PCB diagnostic ion $(M–COCH_3)^+$ clearly indicates a false positive. If the sensitivity is too low to observe these fragment ions, caution should be exercised in identifying the peak. High resolution MS is capable of resolving the molecular ions of MeO–PCBs and CDDs, but a resolution >9,000 must be employed, which is often incompatible with sensitivity requirements.

Application of the method

To illustrate the method, the following samples were analyzed: fish muscle and herring gull egg samples from Lake Ontario which were highly contaminated with a wide variety of organochlorines, a bald eagle egg from a relatively uncontaminated area in Eastern Canada and a great blue heron egg sample from an area contaminated with chlorophenols.

The salmon muscle sample contained 9 mg/kg PCBs, 1.4 mg/kg DDE, 0.3 mg/kg mirex, 0.13 mg/kg of hexachlorobenzene and dieldrin, 0.02 mg/kg of oxychlordane and heptachlor epoxide and minor amounts of other compounds from analysis by electron-capture GC. Figure 3(A) shows the T_4CDD chromatographic window for three ions from the screening analysis of this sample. There were nine peaks with the correct ratio of ions in the molecular ion cluster (including the ones not shown), but the only peak which had the correct relative abundance of $(M–COCl)^+$ (bottom chromatogram) was 2,3,7,8-T_4CDD. The other peaks all had a relatively strong $(M–COCH_3)^+$ ion, indicating that they were probably MeO–PCBs. These compounds have been identified in herring gulls[16] and fish[17] in the Great Lakes area and are known to be metabolites of PCBs.[18] Analysis with the SP2330 column confirmed that only 2,3,7,8-T_4CDD was present, at a level of 80 ng/kg.

Figure 3(B) shows a chromatogram from the quantitative analysis of a herring gull egg sample from Lake Ontario. This sample was also highly contaminated with other organochlorine compounds: 52 mg/kg PCBs, 9 mg/kg DDE, 2.5 mg/kg mirex, and a number of

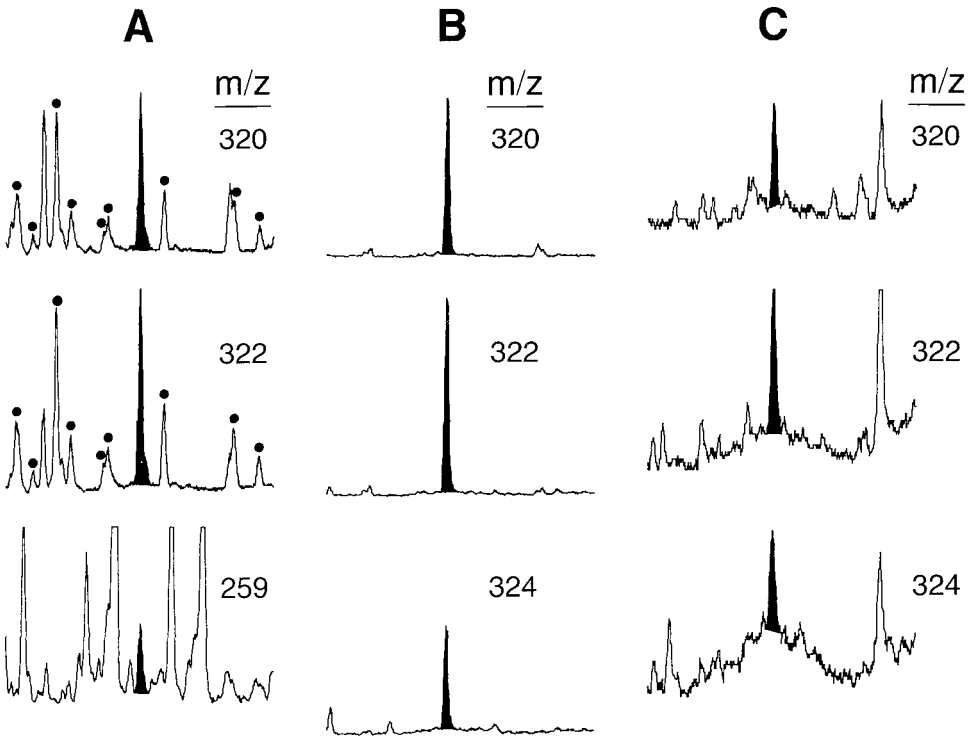

FIGURE 3 Mass chromatograms in the T_4CDD window for: A, Lake Ontario coho salmon muscle, showing two ions in the M^+ cluster and $(M-COCl)^+$, m/z 259; B, Lake Ontario herring gull egg, showing three ions in the M^+ cluster; C, Atlantic coast bald eagle egg, showing three ions in the M^+ cluster. The highlighted peak is 2,3,7,8-T_4CDD in all cases. The peaks in A with dots over them had the correct ratio of ion intensities for three ions in the molecular ion cluster for T_4CDDs, but most clearly had a small or no m/z 259 peak.

compounds in the 0.1–0.3 mg/kg range. In spite of the high PCB level, there was no evidence for the presence of MeO–PCBs, and 2,3,7,8-T_4CDD was unambiguously identified and quantitated at a level of 158 ng/kg.

A peak with nearly the correct retention time for 1,2,3,4,6,7,9-H_7CDD was present in this sample and several others we have analyzed from the Great Lakes. On the SP2330 column this peak changed identity to the other H_7CDD isomer, even though it

appeared to satisfy the criteria for CDD identity based on molecular ion ratios and presence of $(M-COCl)^+$ on both columns. This clearly indicated the presence of an easily overlooked false-positive interference. Further investigation revealed the presence of a strong molecular ion for a MeO–octachlorodiphenylether (MeO–CDPE) isomer. These compounds readily lose CH_3Cl to form a CDD^+ moiety if there is *o*-Cl and *o'*-MeO substitution, and subsequent fragmentation is indistinguishable from that of an authentic CDD,[19] even by high resolution MS. This emphasizes the importance of checking identities on more than one column. Analysis on a DB-17 column (J & W Scientific) was required to clearly indicate that the MeO–CDPE did not co-elute with one of the two H_7CDD isomers.

Figure 3(C) shows a chromatogram from the determination of 2,3,7,8-T_4CDD in the bald eagle egg. This sample had levels of organochlorine contamination similar to those of the salmon muscle and heron egg, even though the female which laid the egg was probably exposed to background levels of contamination. Only the peak corresponding to 2,3,7,8-T_4CDD had the correct ion intensity ratios, and was easily quantified at the 13 ng/kg level.

The great blue heron egg sample contained PCBs and other organochlorine compounds at levels similar to those in the salmon muscle above. Levels of P_5CDDs and H_6CDDs were higher than T_4CDDs, as indicated in Figure 4. In this sample there were no MeO–PCBs or other false-positive interferences, and all CDD isomers were unambiguously identified and quantitated.

SUMMARY

GPC cleanup is an effective means of removing bulk lipids from extracts of biological tissues prior to CDD analysis. Subsequent alumina and Florisil chromatography steps efficiently remove remaining biogenic compounds which may interfere with ng/kg level CDD determination. Non-polar organochlorine compounds such as DDE and most PCB isomers, and highly polar compounds such as hydroxy-PCBs, are also efficiently removed in the column chromatographic steps. The method utilizes only equipment and material readily available to a standard organochlorine analysis laboratory, and is therefore easily implemented for routine use.

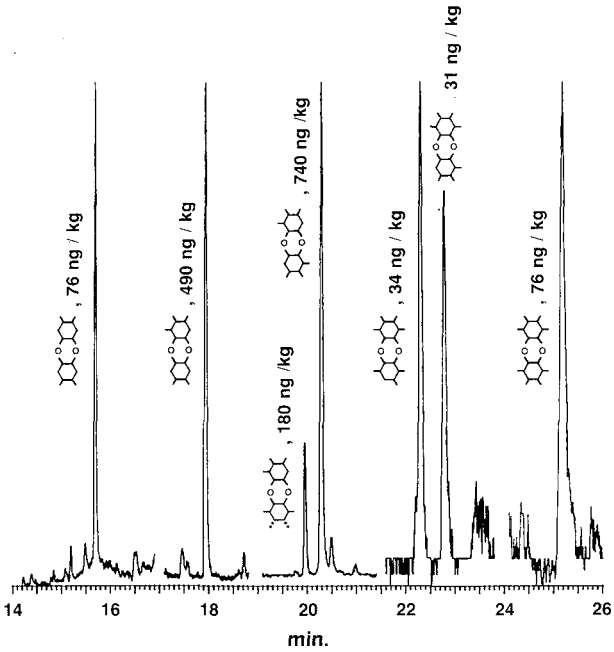

FIGURE 4 Mass chromatogram of a great blue heron egg from the Pacific coast, showing the first ion in the M^+ cluster in each of the T_4CDD to O_8CDD chromatographic windows.

Accuracy and precision of the method at low ng/kg levels is excellent for T_4CDDs–H_7CCDDs except 1,3,6,8- and 1,3,7,9-T_4CDD, which are partially lost in alumina chromatography steps. O_8CDD recovery is lower than that of other CDDs, but inclusion of a $(^{13}C_{12})$–O_8CDD internal standard greatly improves accuracy and precision.

In the samples we have analyzed, interferences which may cause difficulty in CDD determination are all moderately polar, oxygen containing, chlorinated aromatic compounds with the same nominal–mass molecular ion cluster as CDDs, or which fragment to produce a CDD-like moiety in the mass spectrometer. In fish samples from highly-contaminated Lake Ontario, a series of tetrachloro-MeO-PCBs was observed. None of the bird egg samples from this or other areas contained MeO–PCBs, but several con-

tained an octachloro-MeO-CDPE isomer which could easily be confused with a H_7CDD isomer. Criteria other than correct molecular ion cluster ratios are therefore mandatory: presence of $(M–COCl)^+$, correct retention time on two columns of different polarity, and perhaps *absence* of an $(M+CH_3Cl)^+$ ion to exclude the possibility of MeO–CDPE interference.

The highest degree of specificity in separation of CDDs from these types of interferences is probably achieved by a combination of normal and reverse-phase HPLC,[3] or carbon column enrichment.[5,15] The latter has been shown to be superior to methods employing column chromatographic procedures alone[2] and can easily be included in-line after the GPC, as originally suggested by Stalling *et al.*[8] We plan to further improve the specificity of our method by including a carbon/glass-fibre step.

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References

1. W. B. Crummett, *Chemosphere* **12,** 429 (1983).
2. W. C. Brumley, J. A. G. Roach, J. A. Sphon, P. A. Dreifuss, D. Andrzejewski, R. A. Niemann and D. A. Firestone, *J. Agric. Food Chem.* **29,** 1040 (1981).
3. L. L. Lamparski, T. J. Nestrick and R. H. Stehl, *Anal. Chem.* **51,** 1453 (1979).
4. D. Firestone, M. Clower, A. P. Borsetti, R. H. Teske and P. E. J. Long, *J. Agric. Food Chem.* **27,** 1171 (1979).
5. L. M. Smith, D. L. Stalling and J. L. Johnson, *Anal. Chem.* **56,** 1830 (1984).
6. R. J. Norstrom, D. J. Hallett, M. Simon and M. J. Mulvihill, *Chlorinated Dioxins and Related Compounds in the Environment* (O. Hutzinger *et al.*, eds.) (Pergamon Press, Oxford, 1982), pp. 173–181.
7. R. J. Norstrom and M. Simon, *IUPAC Pesticide Chemistry: Human Welfare and the Environment* (J. Miyamoto *et al.*, eds.) (Pergamon Press, Oxford, 1983), pp. 165–170.
8. D. L. Stalling, L. M. Smith and J. D. Petty, *ASTM Special Technical Publication 686* (American Society for Testing Materials, Philadelphia, 1979), pp. 302–323.
9. C. Rappe, S. Marklund, M. Nygren and A. Gara, *Chlorinated Dioxins and Dibenzofurans in the Total Environment* (G. Choudhary *et al.*, eds.) (Butterworth Publishers, Boston, 1983), pp. 259–272.

10. D. L. Stalling, R. J. Norstrom, L. M. Smith and M. Simon, *Chemosphere* **14,** 627 (1985).
11. C. Rappe, P.-A. Bergqvist and S. Marklund, *Chlorinated Dioxins and Dibenzofurans in the Total Environment*, Vol. 2 (G. Choudhary *et al.*, eds.) (Butterworth Publishers, Boston, 1984), pp. 125–138.
12. C. Rappe, P.-A. Bergqvist and S. Marklund, *Analysis of Polychlorinated Dioxins and Dibenzofurans in Aquatic Samples*, Presented at the 3rd Finnish–Swedish Seminar on the Gulf of Bothnia, Pori, Finland, August 20–21, 1984, 9 pp.
13. H. R. Buser and C. Rappe, *Anal. Chem.* **56,** 442 (1984).
14. J. J. Ryan, J. C. Pilon, H. B. S. Conacher and D. Firestone, *J. Assoc. Offic. Anal. Chem.* **66,** 700 (1983).
15. L. M. Smith and J. L. Johnson, *Chlorinated Dioxins and Dibenzofurans in the Total Environment* (G. Choudhary *et al.*, eds.) (Butterworth Publishers, Boston, 1983), pp. 321–332.
16. D. J. Hallett, R. J. Norstrom, F. I. Onuska and M. E. Comba, *Fate of Pesticides in Large Animals* (G. W. Ivie and H. W. Dorough, eds.) (Academic Press, N.Y., 1977), pp. 183–192.
17. D. W. Phillipson and B. J. Puma, *Anal. Chem.* **52,** 2328 (1980).
18. J. P. Lay, W. Klein and F. Korte, *Chemosphere* **4,** 161 (1975).
19. M. T. M. Tulp and O. Hutzinger, *Biomed. Mass Spectrometry* **5,** 35 (1978).