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Dual-column high-performance liquid chromatographic cleanup procedure for the determination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans in fish tissue

T. S. THOMPSON*[,a], T. M. KOLIC and K. A. MacPHERSON

Ministry of the Environment, Dioxin Laboratory, Laboratory Services Branch, 125 Resources Road, P.O. Box 213, Rexdale, Ontario M9W 5L1 (Canada)

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ABSTRACT

A high-performance liquid chromatographic cleanup procedure employing normal-phase alumina and carbon–silica separations was developed for isolating polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) from other polychlorinated pollutants present in fish tissue. The method utilizes a column-switching step where the dioxins and furans are trace enriched onto a carbon–silica column as they are eluted from the alumina column. Interfering components such as polychlorinated biphenyls and chlorinated diphenyl ethers elute through the carbon–silica column. The PCDDs and PCDFs are subsequently recovered by backflushing the carbon–silica column using toluene.

INTRODUCTION

The determination of low parts-per-trillion (ppt)[b] concentrations of chlorinated dioxins and furans in fish tissue is complicated by the presence of relatively large amounts of other polychlorinated pollutants. Compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCs), and other chlorinated aromatic hydrocarbons are widespread in the environment. Like dioxins and furans, PCBs and OCs tend to accumulate in biota, however, they are generally found at concentrations several orders of magnitude higher than the polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs). Polychlorinated naphthalenes and diphenyl ethers (PCNs and PCDPEs, respectively) may also be present at levels much higher than either the dioxins or furans.

The presence of PCDPEs poses a major problem in the determination of PCDFs by gas chromatography (GC)–mass spectrometry (MS) techniques since these compounds rearrange under electron impact ionization conditions to yield ions corresponding to PCDF molecular ions. Therefore PCDPEs will interfere in the determination of PCDFs regardless of whether the analysis is carried out using

[a] Present address: Saskatchewan Health, Laboratory and Disease Control Services Branch, H. E. Robertson Laboratory, 3211 Albert Street, Regina, Saskatchewan S4S 5W6, Canada.

[b] Throughout the article the American trillion (10^{12}) is meant.

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low-resolution, high-resolution, or tandem mass spectrometry (LRMS, HRMS or MS–MS). PCBs strongly interfere in the determination of PCDDs by LRMS and even HRMS if they are present at several orders of magnitude higher in concentration than the dioxins. While MS–MS very successfully eliminates the interference due to PCBs by selectively monitoring the loss of a COCl group from the PCDD molecular ion, it is still desirable to remove the bulk of the PCBs present in the sample extract. It should also be noted that high concentrations of PCBs and other components may affect response factors involved in the quantitation of PCDDs and PCDFs using either LRMS or HRMS.

In order to unambiguously identify and quantitate low ppt levels of PCDDs and PCDFs in fish tissue, highly efficient sample cleanup procedures must be employed. Alumina has been identified as the most frequently used adsorbent in sample preparation procedures for the determination of dioxins and furans in various sample matrices [1]. PCDDs and PCDFs may be separated from other closely related polychlorinated pollutants using alumina liquid–solid chromatography [2–5]. Activated carbon is also frequently used in the isolation of dioxins and furans. Careful selection of mobile phase composition permits fused ring aromatic compounds such as PCDDs, PCDFs, and PCNs to be retained while non-planar compounds such as PCBs and polychlorinated diphenyl ethers are eluted [6,7].

Since early work by Baughman and Meselson [8], numerous cleanup techniques have been employed by various groups. Many of the sample preparation procedures reported in the literature are variations of the method initially devised by Lamparski *et al.* [9]. Acid-, base- and silver nitrate-modified silica gel packing materials and alumina were used to remove the bulk of the chemical interferences from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Final isolation of 2,3,7,8-TCDD was accomplished using reversed-phase high-performance liquid chromatography (HPLC).

HPLC is increasingly being used as a sample preparation technique in environmental analysis [10–12]. Despite the frequent use of alumina in open column liquid–solid chromatographic separations, relatively little work has been done utilizing alumina as an HPLC stationary phase. The major reason behind the lack of success in employing alumina normal-phase HPLC as a cleanup technique is the difficulty in obtaining a constant and reproducible activity. Dolphin initially demonstrated that dioxins could be separated from PCBs, PCNs, 2,2-bis-(4-chlorophenyl)-1,1-dichloroethene (*p,p'*-DDE) and 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane (*p,p'*-DDT) [13]. However in this study and a subsequent one [14], the separation of PCDDs from the other polychlorinated pollutants was demonstrated using only standard solutions. It was not until recently that a practical application of alumina HPLC for the cleanup of environmental samples was demonstrated [15]. Thompson devised a method for isolating 2,3,7,8-TCDD from compounds co-extracted from fish tissue using two HPLC separation procedures, first on silica and finally on alumina. The use of C_{18} reversed-phase and alumina normal-phase HPLC fractionation procedures for the isolation of PCDDs and PCDFs from co-extracted compounds was also demonstrated [16].

O'Keefe *et al.* [17] developed a semi-automated cleanup method for the determination of PCDDs and PCDFs in environmental samples. Sample extracts are fractionated on acidic alumina, Amoco PX-21 carbon dispersed on Celite 545, and neutral alumina in a serial process. By using a combination of switching valves, solvent

reservoirs, and a low-pressure pump the system was semi-automated. Donnelly reported the use of AX-21 carbon dispersed on silica as an HPLC stationary phase for the cleanup of environmental samples [18]. This carbon HPLC cleanup step was performed after initial cleanup using multi-phase silica and alumina columns.

In the work presented herein, the liquid chromatographic fractionation steps are carried out using a single HPLC separation procedure employing both alumina and carbon stationary phases. A series of eighteen fish tissue samples were processed using the HPLC cleanup procedure.

EXPERIMENTAL

Initial preparation of fish tissue samples

Each tissue sample consists of a homogenized composite sample of whole small fish. Approximately 20 g of ground fish is weighed into a 250-ml erlenmeyer flask. Each sample is spiked with a standard solution containing five $^{13}C_{12}$-labelled dioxin isomers (one per congener group for each of tetra- through octachlorodioxin). The levels of the individual isotopically labelled internal standards are given in Table III. Approximately 75 ml of hydrochloric acid, previously extracted with dichloromethane followed by hexane, is added to each flask. The acid digestion is allowed to proceed overnight (about 16 h).

The acid digest is extracted with three 70-ml portions of hexane using a 250-ml separatory funnel. Each hexane extract is passed through a cylindrical funnel (14 × 3.5 cm I.D.) which contains a 2-cm layer of anhydrous sodium sulphate over a 4-cm layer of a 44% (w/w) mixture of sulphuric acid and silica gel. By passing the hexane extracts through this column, residual water and oily co-extracted material are removed. After all three hexane portions have been collected, the separatory funnel is rinsed with a fresh aliquot of hexane and the rinsing is passed through the sodium sulphate/acid–silica column. The extracts are concentrated by rotary evaporation under vacuum with the aid of a warm water bath. Each extract is quantitatively transferred to a 100-μl conical glass vial and concentrated just to dryness under a gentle stream of ultrahigh purity nitrogen gas. The final residue is reconstituted with 30 μl of hexane prior to injection on the HPLC system.

High-performance liquid chromatography cleanup

All HPLC separations are performed using a Waters high-performance liquid chromatography system. This system consists of three Model 510 dual piston pumps (only two are required) and a Waters 481 Lambda Max variable-wavelength ultraviolet detector. The pumps and detector are controlled by an NEC APCIV personal computer to which they are linked via the Waters System Interface Module. This permits complete computerized control of the system in addition to data storage and manipulation. Sample extracts are introduced into the HPLC using a Rheodyne 7125 injector system equipped with a 50-μl sample loop. All initial separations are achieved using a normal-phase alumina column (0.46 cm I.D. × 25; Spherisorb 5-μm particulates from Phenomenex, Torrance, CA, U.S.A.). Secondary separations are performed using a carbon–silica column prepared in our laboratory. A mixture of 10% (w/w) of Amoco PX-21 carbon and silica gel (70–230 mesh) was prepared and packed into a empty Waters guard column (3 × 0.46 cm I.D.). The silica does not provide any additional separation but rather it serves as a solid support for the carbon. The

carbon–silica mixture was activated at 130°C overnight prior to being packed into the guard column. Once packed into the column, the carbon–silica mixture was washed with approximately 1 l of toluene before being used.

Two four-port pneumatically actuated valves (Valco, Houston, TX, U.S.A.) are used to permit the column-switching and backflushing steps. A Tracor 955 LC pump is used to backflush the carbon–silica column with toluene. A general schematic diagram of the entire HPLC system is illustrated in Fig. 1. The gradient elution program and valve switching are described in Table I.

After trace enriching the dioxins and furans onto the carbon–silica column, the PCDDs and PCDFs are eluted by backflushing with 75 ml of toluene. The toluene extracts are concentrated by rotary evaporation and transferred to conical vials where they are taken just to dryness by nitrogen gas blowdown. Prior to being analyzed by GC–MS, the residues are reconstituted in 20 μl of toluene.

Once a run has been completed, the system must be returned to its initial state. The column-switching valve (1 in Fig. 1) is switched to position 2 and dichloromethane followed by hexane are pumped through both the alumina and carbon–silica columns for approximately 20 min each. When the absorbance reading on the UV detector returns to zero, the toluene and any other UV adsorbing material have been completely flushed from the carbon–silica column and the system is ready for the next sample injection.

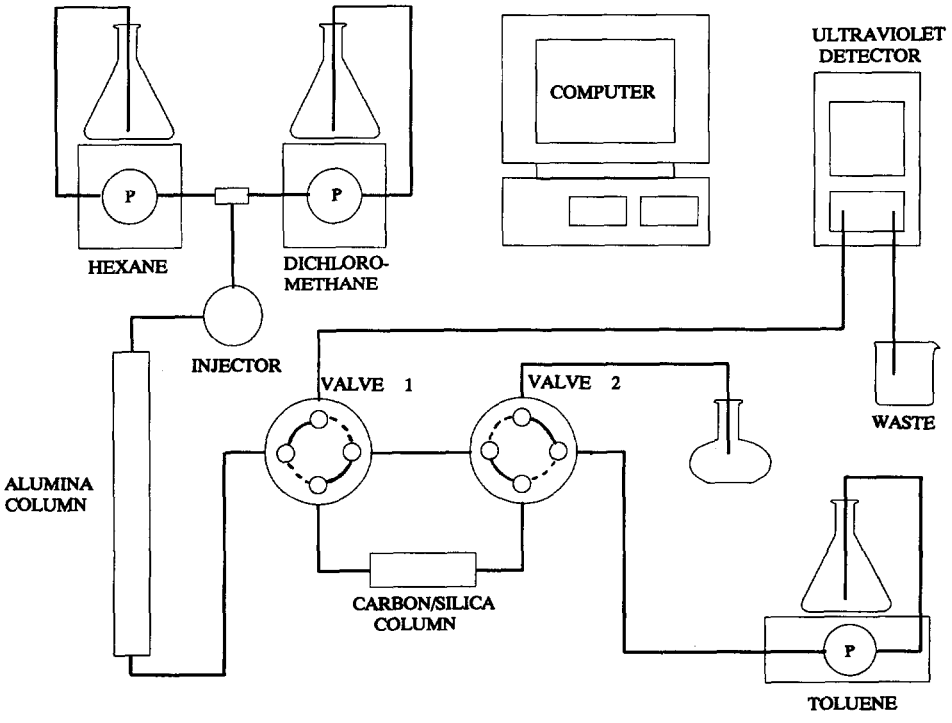

Fig. 1. Schematic diagram of HPLC system used for cleanup of fish tissue extracts. P = HPLC pump; ——— = valve position 1; – – – = valve position 2.

TABLE I

HPLC GRADIENT ELUTION PROGRAM AND VALVE CONFIGURATION

V1 = valve 1 in Fig. 1 (1 = position 1, 2 = position 2); V2 = valve 2 in Fig. 1 (1 = position 1, 2 = position 2); B.F. pump = secondary pump used for backflushing carbon column.

Time (min)	Primary pumping system			V1	V2	B.F. pump flow-rate (ml/min)
	Flow-rate (ml/min)	% Hexane	% Dichloromethane			
0	2.0	100	0	1	1	0
10	2.0	100	0	1	1	0
12	2.0	90	10	2	1	0
15	2.0	75	25	2	1	0
35	2.0	75	25	1	2	0
36	2.0	60	40	1	2	5
40	2.0	0	100	1	2	5
51	2.0	0	100	1	1	5
52	2.0	0	100	1	1	0

GC–MS determination of PCDDs and PCDFs

All GC–MS analyses are carried out using a Finnigan 4500 gas chromatograph–mass spectrometer. The GC system is linked to the low-resolution quadrupole mass spectrometer by a direct capillary interface. A cool on-column injection system and 30-m DB-5 fused-silica capillary column (0.25 mm I.D. with 0.25 μm film thickness; J & W Scientific, Folsom, CA, U.S.A.) are employed in all analyses. The oven

TABLE II

GC–MS SIM PARAMETERS

Compound[a]	Ions monitored	Group No.	Compound[a]	Ions monitored	Group No.
TCDFs	304, 306, 308	1	H_7CDFs	406, 408, 410	4
TCDDs	320, 322, 324	1	H_7CDDs	422, 424, 426	4
$^{13}C_{12}$-TCDD	332, 334	1	$^{13}C_{12}$-H_7CDD	436, 438	4
P_5CDPE	340	1	OCDPE	442	4
H_6CDPE	374	1	N_9CDPE	476	4
P_5CDFs	338, 340, 342	2	OCDF	442, 444, 446	5
P_5CDDs	354, 356, 358	2	OCDD	458, 460, 462	5
$^{13}C_{12}$-P_5CDD	368, 370	2	$^{13}C_{12}$-OCDD	470, 472	5
H_6CDPE	374	2			
H_7CDPE	408	2			
H_6CDFs	372, 374, 376	3			
H_6CDDs	388, 390, 392	3			
$^{13}C_{12}$-H_6CDD	402, 404	3			
H_7CDPE	408	3			
OCDPE	442	3			

[a] T = tetra, P_5 = penta, H_6 = hexa, H_7 = hepta, O = octa, N_9 = nona.

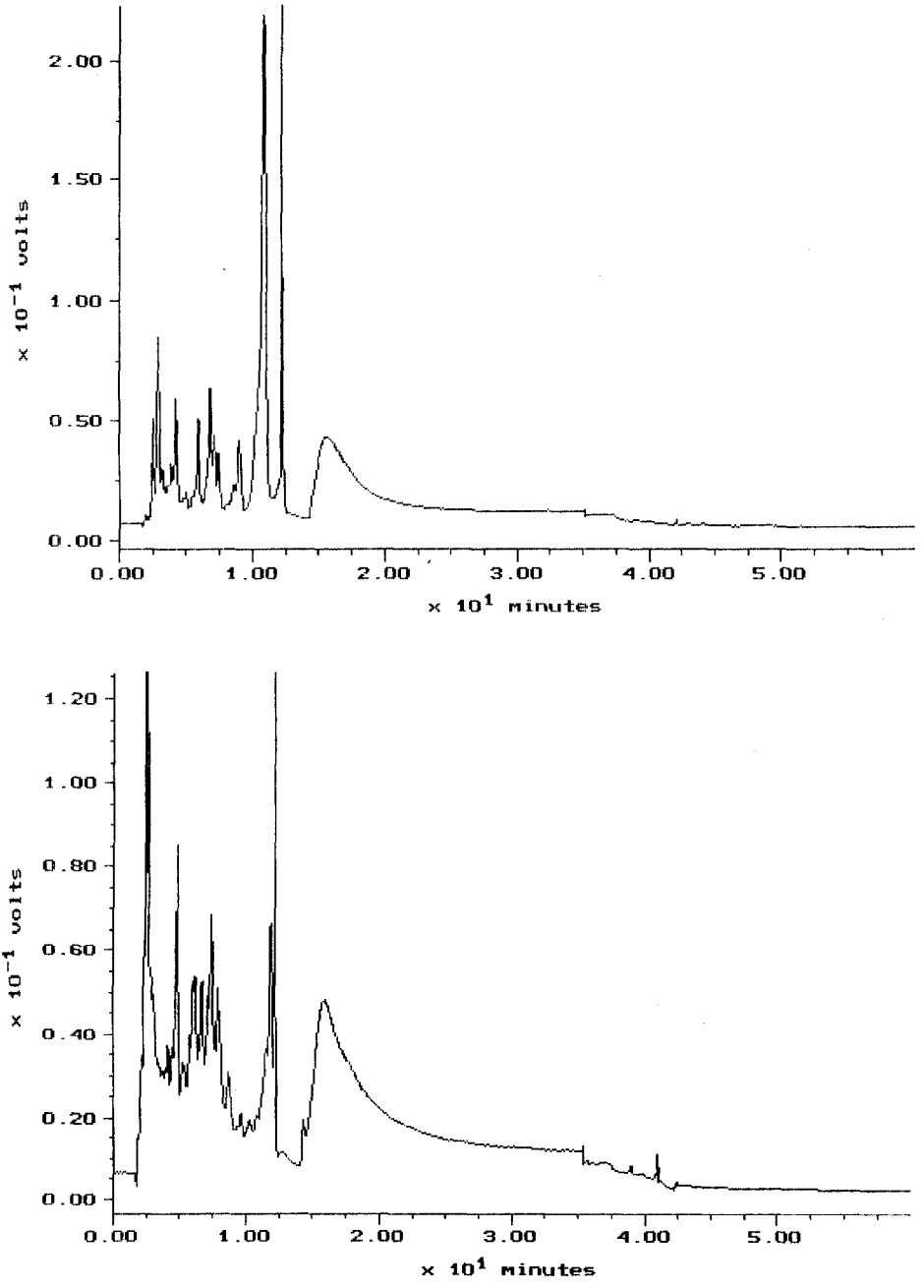

Fig. 2. HPLC chromatograms (UV detection at 254 nm) for fish tissue extracts.

temperature program is: initial oven temperature held for 2 min at 110°C, ramped to 250°C at 15°C/min, ramped to 300°C at 5°C/min and held for 5 min. Helium is used as the GC carrier gas with a column head pressure of 16 p.s.i.

The determination of PCDDs and PCDFs is typically accomplished using selected ion monitoring (SIM) techniques. Three molecular ions are monitored for each congener group in addition to two ions for each $^{13}C_{12}$-labelled internal standard. Two additional ions corresponding to chlorinated diphenyl ethers are also monitored for each congener group (except octachloro) to ensure that no PCDPE interferences are observed. The ions monitored are summarized in Table II. All GC–MS analyses are performed using an electron energy of 35 eV and an electron multiplier voltage of 1200 V.

RESULTS AND DISCUSSION

A series of eighteen fish tissue samples were prepared using the extraction and cleanup procedure described in the experimental section. Fig. 2 shows the HPLC chromatograms (UV detection at 254 nm) which were obtained for two of the fish samples. In both chromatograms, which were typical of most of the samples analyzed, there appears to be a significant amount of material which elutes in the first twelve minutes of the run. These components were most likely PCBs which have been found to largely elute between 3 and 15 minutes on the alumina column using this particular gradient elution program. The later eluting components may have included some

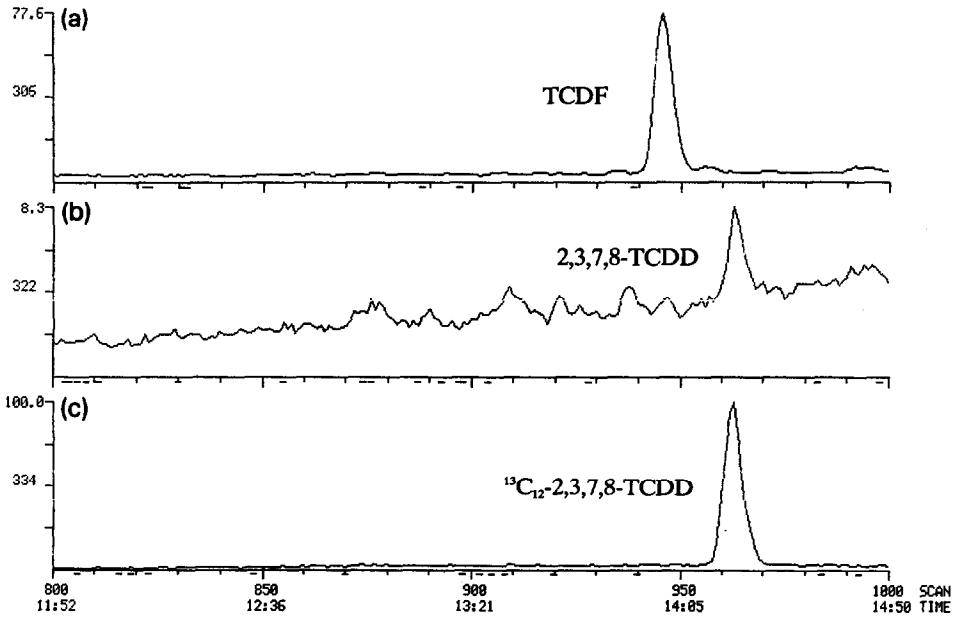

Fig. 3. Reconstructed ion chromatograms for (a) 160 ppt TCDF and (b) 10 ppt 2,3,7,8-TCDD in fish tissue. (c) Reconstructed ion chromatogram for $^{13}C_{12}$-2,3,7,8-TCDD internal standard. Time in min:s. The *y*-axes show the *m/z* values and the intensities (%).

PCDPEs, compounds that are frequently encountered in fish tissue samples collected in or around the Great Lakes.

The reconstructed ion chromatograms obtained for the samples processed using the HPLC cleanup scheme were found to be free of interferences. This permitted the unambiguous identification and quantitation of dioxins and furans at the low ppt level. No PCDPE interferences were observed for any of the samples analyzed. PCBs also appear to have been effectively removed using the dual column HPLC cleanup procedure. Figs. 3 and 4 show the reconstructed ion chromatograms obtained for the determination of TCDDs and TCDFs in two of the tissue samples analyzed. The concentrations of 2,3,7,8-TCDD in the two samples were 10 and 14 ppt (Figs. 3 and 4, respectively) while the concentrations of the TCDF were 160 and 91 ppt. No carry over was observed in any of the samples cleaned using the HPLC method. Both samples run after the samples containing 91 and 160 ppt of TCDF were found to have nondetectable levels of TCDF.

In order to investigate the reproducibility of the extraction and HPLC cleanup, the mean and standard deviation for the recovery of the individual isotopically labelled internal standards were calculated. The mean recoveries along with their respective standard deviations and relative standard deviations are given in Table III. The mean recoveries were found to range from 48 to 63% with typical relative standard deviations (R.S.D.) being approximately 28%.

Table IV shows the concentration ranges of the dioxins and furans found in the fish tissue samples. Mean detection limits were calculated based on those samples for which no dioxins or furans were detected. It should be noted that the results for two of

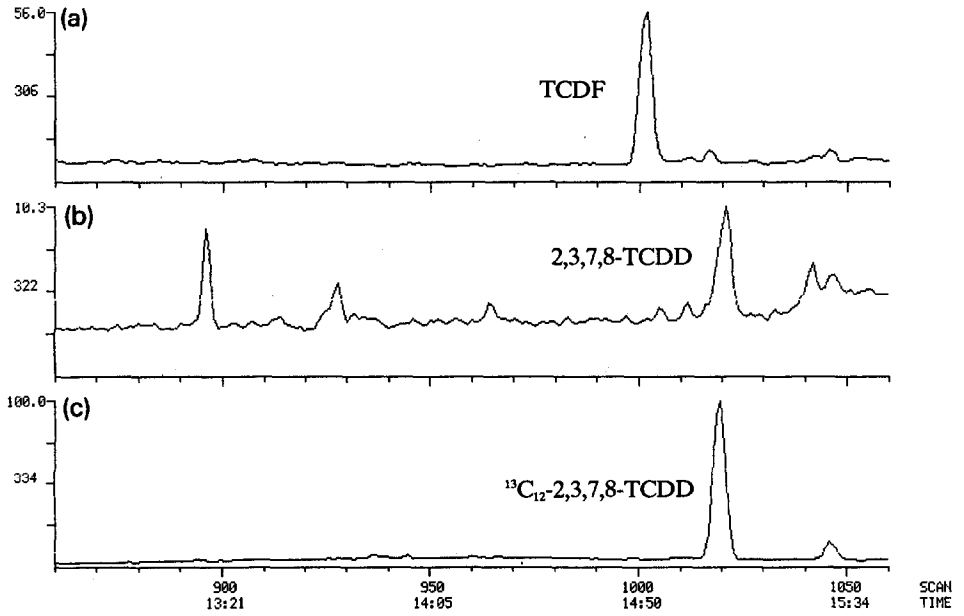

Fig. 4. Reconstructed ion chromatograms for (a) 91 ppt TCDF and (b) 14 ppt 2,3,7,8-TCDD in fish tissue. (c) Reconstructed ion chromatogram for $^{13}C_{12}$-2,3,7,8-TCDD internal standard. Time in min:s. The *y*-axes show the *m/z* values and the intensities (%).

TABLE III

RECOVERY OF ISOTOPICALLY LABELLED INTERNAL STANDARDS

Calculations based on results obtained from eighteen fish tissue samples.

Isomer	Level of spike (ng)	Mean recovery ± S.D. (%)	% R.S.D.
$^{13}C_{12}$-2,3,7,8-TCDD	4.9	48 ± 14	29
$^{13}C_{12}$-1,2,3,7,8-P_5CDD	5.5	63 ± 17	27
$^{13}C_{12}$-1,2,3,4,7,8-H_6CDD	4.1	56 ± 15	26
$^{13}C_{12}$-1,2,3,4,6,7,8-H_7CDD	18.8	57 ± 18	31
$^{13}C_{12}$-OCDD	9.2	62 ± 15	25

the fish tissue samples were not included in these calculations. The sample weights (3 to 4 grams) were much smaller than the 20 grams used for the majority of the samples which resulted in considerably higher detection limits.

CONCLUSIONS

The HPLC cleanup method devised in this study was found to be very effective in removing interferences such as PCBs and PCDPEs. The recovery of internal standards was found to be acceptable and quite consistent (average relative standard deviations of 28%). This procedure permitted the final analysis to be performed using a LRMS system. More selective techniques such as HRMS or MS–MS were not required because no interferences were encountered, however the increased sensitivity of such instrumentation would result in much lower detection limits.

TABLE IV

SUMMARY OF PCDD AND PCDF RESULTS

Based on results from sixteen fish tissue samples (two samples with only 3 to 4 g total weight were omitted in this summary). Superscripts indicate the number of isomers detected for each congener group. ND = Not detected. DL = Detection limit.

Congener group	Number of samples with positives	Concentrations (ppt)	Number of NDs	Mean DL (ppt)
2,3,7,8-TCDD	4	10, 14, 15, 27	12	4
TCDD	4	10^1, 14^1, 23^2, 66^2	12	4
P_5CDD	1	3^1	15	6
H_6CDD	1	22^2	15	8
H_7CDD	1	130^2	15	7
OCDD	2	15, 380	14	7
TCDF	5	7^1, 25^1, 60^1, 91^1, 160^1	11	4
P_5CDF	0		16	4
H_6CDF	1	36^2	15	6
H_7CDF	1	55^2	15	8
OCDF	1	44	15	6

This cleanup procedure should be easily automated and at this time we are currently setting up a fully automated HPLC system complete with autosampler, fraction collector, and automated switching valves. With such an arrangement we hope to be able to process a set of 10 tissue samples (and associated quality assurance/quality control (QA/QC) samples) through the HPLC cleanup in about 20 h.

In future work, we will be evaluating the useful lifetime of the carbon–silica column. No deterioration of performance was observed during the injection of numerous standard solutions, QA/QC samples, and the 18 tissue sample extracts. By using the column-switching step, the bulk of the pollutants are not introduced onto this column which presumably extends its useful lifetime.

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