NWRI Contribution No. 89-133

A PROCEDURE FOR THE DETERMINATION OF POLYCHLORINATED DIBENZO-P-DIOXINS IN FISH.

by

J.P. Sherry*[1] and H. Tse[2]

[1]Research and Applications Branch, National Water Research Institute, CCIW, 867 Lakeshore Rd., Burlington, Ontario L7R-4A6.
[2]National Water Quality Laboratory, CCIW, 867 Lakeshore Rd., Burlington, Ontario L7R-4A6.

MANAGEMENT PERSPECTIVE

The determination of polychlorinated dibenzo-p-dioxins (PCDDs) in fish is a time consuming and laborious process. Method developments that improve analytical efficiency without compromising performance will benefit both routine and research programs. With that objective, some potential improvements to a previously used method for the analysis of PCDDs were investigated. A neutral solid phase extraction technique yielded equivalent results to a laborious and time consuming liquid phase acidic extraction technique. A multi-layer clean-up column, which replaces three separate liquid phase clean-up steps, was developed and evaluated with satisfactory results. The improved method was evaluated using a variety of fish samples. The enhanced efficiency and effectiveness of the described method will benefit the Directorate's PCDD surveillance and monitoring programs.

Dr. J. Lawrence
Director
Research Applications Branch

PERSPECTIVE-GESTION

Le dosage des polychlorodibenzo-p-dioxines (PCDD) dans le poisson demande beaucoup de temps et beaucoup d'effort. Les améliorations qui permettront d'accroître l'efficacité des méthodes d'analyse sans pour autant en diminuer la performance seront avantageuses tant dans les programmes d'analyse de routine que dans les programmes de recherche. C'est avec cet objectif en tête que nous avons examiné certaines améliorations susceptibles d'être appliquées à une méthode qui servait auparavant au dosage des PCDD. Une technique d'extraction en phase solide neutre a donné des résultats équivalents à ceux obtenus avec une technique d'extraction en phase liquide acide qui était à la fois longue et laborieuse. Nous avons mis au point une colonne de purification à couches multiples qui permet de remplacer trois étapes distinctes de purification en phase liquide; l'évaluation de cette colonne a révélé qu'elle donnait des résultats satisfaisants. La méthode améliorée a été évaluée avec différents échantillons de poisson. L'efficacité accrue de la méthode décrite sera utilisée avantageusement dans le cadre des programmes de surveillance et de contrôle des PCDD mis en oeuvre dans la Direction générale.

M. J. Lawrence

Directeur

Direction de la recherche et des applications

ABSTRACT

Some approaches designed to improve the efficiency of a method for the determination of polychlorinated dibenzo-p-dioxins in fish, without compromising performance, were explored. A solid phase extraction method was compared with aqueous phase acidic extraction: ^{13}C-PCDD recoveries were comparable (>95%) for both methods. An effective multi-layer clean-up column was less time consuming than previously used liquid phase acid/base treatments. Using the modified method, ^{13}C-T_4CDD recoveries were acceptable at low (10ppt), medium (20ppt), and high (100ppt) spiking levels; recoveries of ^{13}C-P_5CDD through ^{13}C-O_8CDD, which were determined at higher levels (2x for P_5CDD - P_7CDD; 3x for O_8CDD), were also satisfactory. The modified method was evaluated using a variety of fish samples; the results indicated acceptable performance: ^{13}C-T_4CDD recoveries were $\geq$ 75%.

Nous avons étudié certaines techniques conçues dans le but d'améliorer l'efficacité, mais sans diminuer la performance, d'une méthode de dosage des polychlorodibenzo-p-dioxines dans le poisson. Nous avons comparé une méthode d'extraction en phase solide à une méthode d'extraction en phase aqueuse acide; dans les deux cas, les taux de récupération de ^{13}C-PCDD étaient comparables (> 95 %). La purification sur une colonne efficace à couches multiples était plus rapide que la méthode par traitement acide/base en phase liquide qui était employée auparavant. Avec la méthode modifiée, les taux de récupération de ^{13}C-PCDD étaient acceptables à des concentrations de dopage faibles (10 ppt*), moyennes (20 ppt*) et élevées (100 ppt*); les taux de récupération des congénères ^{13}C-P_5CDD à ^{13}C-O_8CDD, qui ont été déterminés à des concentrations de dopage plus élevées (2x pour les congénères P_5CDD à P_7CDD et 3x pour les congénères O_8CDD), étaient également satisfaisants. La méthode modifiée a été évaluée avec différents échantillons de poisson; les résultats obtenus révèlent que la performance est acceptable, les taux de récupération du ^{13}C-T_4CDD étant ≥75 %.

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*ppt = parties par billion (10^{12})

1.0 INTRODUCTION

The determination of polychlorinated dibenzo-p-dioxins (PCDDs) in environmental samples is costly and time consuming. The identification, in recent years, of additional environmental inputs, such as pulp and paper mill effluents (Swanson et al. 1988; Clement et al., 1989), and the reported contamination of some food and consumer products with PCDDs (NRCC, 1981; Firestone et al., 1986; Beck et al., 1987, 1989; Ryan et al., 1988;) has led to a dramatic increase in the number and variety of samples requiring dioxin analysis. Moreover, the contamination from known sources must be quantified and monitored as the quest for further sources and problem areas continues.

Screening techniques, such as immunoassays, have the potential to reduce analytical overloads. However, a previous study (Sherry et al., 1989) indicated that considerable sample clean-up was required before fish extracts could be effectively analyzed by radioimmunoassay (RIA). The extraction and clean-up method (Afghan et al., 1987) used in that study was effective but unwieldy. With the objective of decreasing sample preparation time, neutral solid-phase extraction was compared with the previously used acidic extraction technique. A multi-layer clean-up column for the single step removal of several interference types was compared with liquid phase acid/base treatments. The resulting method was evaluated using a variety of fish samples.

2.0 MATERIALS AND METHODS

2.1 Fish Matrices

A Lake Trout (Salvelinus namaycush) (2.8 Kg, 20% lipid), taken from a polluted section of the eastern basin of L. Ontario, was used in the main experiments. Carp (Cyprinus carpio Linnaeus-), and Walleye (Stizostedion vitreum) were also used.

2.2 Spiking and Extraction Procedures

A portion of spiking solution, containing a mixture of surrogates (^{13}C-2,3,7,8-T4CDD; ^{13}C-1,2,3,7,8-P_5CDD; ^{13}C-1,2,3,4,-7,8-H_6CDD; ^{13}C-1,2,3,4,6,7,8-H_7CDD; ^{13}C-O_8CDD) in toluene, was spread over a shallow depression on the surface of the whole fish homogenate, and allowed to soak in for 1 h.

2.2.1 Solid Phase (neutral; selected procedure).

Fish homogenate (10 or 25 g) was thoroughly ground in anhydrous Na_2SO_4 (200 g); the resulting mixture was transferred to a glass column (3 cm i.d. * 50 cm). The column was eluted with 800 mL of dichloromethane (DCM):hexane 1:1.

2.2.2 Liquid Phase (acidic).

Fish tissue (10 g) was digested in a 500 mL Erlenmeyer flask, using 200 mL of 6 M HCl in toluene (1:1); the mixture was vigorously agitated using a wrist action shaker (12 h). Organic and aqueous phases were separated by centrifugation,

and the aqueous phase was re-extracted twice with toluene (Afghan et al., 1987).

2.3 Bulk Lipid Removal

After solvent removal, the extract was re-suspended (0.2 g/mL) in DCM:hexane (1:1) and clarified by filtration (5.0 um). The extract was chromatographed on Bio Beads S-X3 (60 g in a 2.5 cm * 60 cm column) using a GPC Autoprep unit (ABC Laboratories Inc.). The column was eluted (DCM:hexane (1:1)) at a flow rate of 5 mL/min.; the first 150 mL was discarded; PCDDs were eluted in the second 150 mL.

2.4 Additional Clean-up Steps

2.4.1 Liquid Phase Acid/Base Treatments.

The eluent from the GPC step was treated with base (0.05M Na_3PO_4) and acid (10N H_2SO_4) as described by Afghan et al., 1987.

2.4.2 Multi-layer Column (selected procedure).

Acidic silica 40% (w/w) was prepared by the stepwise addition of concentrated H_2SO_4 to activated silica gel. Similarly, basic silica 30% (w/w) was prepared using 1.5 M KOH, and $AgNO_3$ treated silica (10% w/w) was prepared using a solution of $AgNO_3$. The eluent from the GPC step was applied to the column

(Fig. 1) in 5 mL of 1% (v/v) toluene in hexane: toluene was included to minimize losses of O_8CDD. The column was eluted with 150 mL of 1% toluene in hexane.

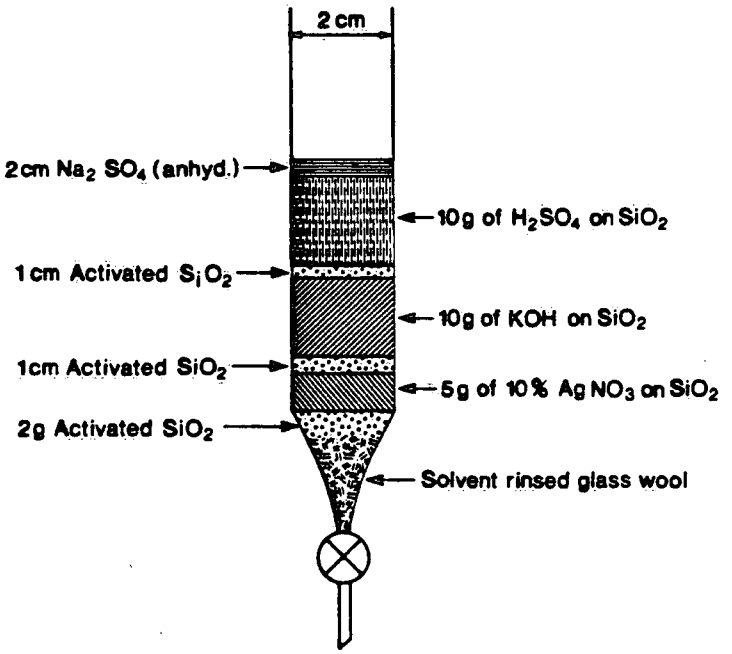

Fig. 1 Multi-layer clean-up column

2.4.3 Alumina and Carbon Fibre Chromatography.

Deactivated (1% w/w) basic alumina (Fisher) was packed (30 g) in a 2.5 cm i.d. glass column. Extract was applied to the column in 1% DCM in hexane; the column was eluted with 200 mL of the same solvent, and then with 150 mL of 50 % DCM in hexane. The latter fraction was transferred to 1 mL of DCM:cyclohexane (1:1), and chromatographed on activated carbon fibre

(Amoco PX 21 on shredded glass fibres (Afghan et al., 1987). PCDDs were recovered from the carbon fibre column by elution with toluene.

2.5 Quantitative Analysis (GC/MSD):

The enriched extract was re-suspended in toluene (25 uL) containing ^{13}C-1,2,3,4-T_4CDD (50 pg/uL), and chromatographed (1 uL) on a 30 m HP-Ultra-2 capillary column using a Hewlett Packard gas chromatograph (GC) (HP5990A). The GC was coupled to a HP 5970 mass selective detector (MSD) (electron impact mode at 70 eV) operated in single ion monitoring mode. Quantification was based on the most intense ion in the molecular clusters; confirmation was based on the presence of at least one other cluster ion; the M - COCl ion was used to confirm native congeners. All ions were required to have the correct retention time, an acceptable peak shape, and the correct mass ratio. The instrument calibration standard, which contained the surrogates, and the corresponding unlabelled PCDD/PCDF isomers, was adjusted to match the samples' surrogate levels, and analyzed before and after every 3 samples. Native isomers and homologues were automatically corrected for the recovery of the corresponding ^{13}C-PCDD surrogate, and were quantified using the relative response of the corresponding surrogate and unlabelled congener in the calibration standard.

3.0 RESULTS

3.1 Preliminary Evaluation:

The initial experiment was a replicated (n=3) comparison of liquid phase acidic extraction (Methods #1 - #4; (Table 1)) with solid phase neutral extraction (Methods #5 - #8; (Table 1)), using lake trout samples (10 g). As outlined in Table 1, an evaluation of several modifications to the reference clean-up procedure (Method #1) was incorporated into the experiment. The vortex (Method #2) and shake (Method #1) extraction techniques yielded similar surrogate recoveries (Table 2). Because of its gentler nature, vortex extraction reduces the risk of flask breakage or extract spills. The acidic (Methods #1 and #2) and neutral (Method #5) extraction techniques yielded comparable surrogate recoveries, when combined with liquid phase acid/base clean-up (Table 2). The limited data (n=3) indicates that acidic extraction was somewhat more precise than neutral extraction for the ^{13}C-P_5CDD - ^{13}C-H_7CDD congeners; however, the methods were equally precise for ^{13}C-T_4CDD, and the CV% for ^{13}C-O_8CDD was apparently lower for the neutral extraction method.

Substitution of the solid phase chromatographic clean-up (SPC) step for the liquid phase acid/base clean-up (LPC) steps caused no apparent deterioration in surrogate recoveries, with either the acidic (Methods #1 (LPC) and #3 (SPC)) or neutral

(Methods #5 (LPC) and #8 (SPC)) extraction procedures (Table 2). With the exception of the ^{13}C-T_4CDD data for Method #8, solid phase clean-up was at least comparable in precision to liquid phase clean-up. Further experiments (Tables 3-5) demonstrated that the ^{13}C-T_4CDD data for Method #8 (Table 2) were atypical.

Elimination of the alumina chromatography step permitted gross interferences to pass through the clean-up, with the consequent loss of the ^{13}C-T_4CDD data (Table 2). Elimination of the acid/base clean-up steps (Method #6) was associated with erratic surrogate recoveries and imprecision (Table 2). Based on the foregoing data, Method #8 was selected for further evaluation.

TABLE 1. Description of Evaluated Methods

	Method							
	1	2	3	4	5	6	7	8
6NHCl shake ex.	+	—	+	+	—	—	—	—
6NHCl vortex ex.	—	+	—	—	—	—	—	—
Neutral column ex.	—	—	—	—	+	+	+	+
GPC	+	+	+	+	+	+	+	+
Na_3PO_4 wash	+	+	—	—	+	—	—	—
H_2SO_4 wash	+	+	—	—	+	—	—	—
Multi-layer column	—	—	+	+	—	—	+	+
Basic alumina	+	+	+	—	+	+	—	+
Carbon fibre	+	+	+	+	+	+	+	+

TABLE 2. SURROGATE RECOVERIES USING A VARIETY OF METHODS

13C12 CONGENER	LEVEL (pg/g)	SAMPLE PREPARATION METHOD 1	2	3	4	5	6	7	8
T4CDD	125	94[1] (12)[2]	98 (1)	117 (13)	(3)	89 (12)	84 (50)	(3)	150 (21)
P5CDD	250	92 (11)	106 (3)	98 (6)	130 (15)	100 (17)	95 (25)	94 (14)	105 (10)
H6CDD	250	106 (11)	109 (1)	104 (7)	123 (16)	105 (19)	107 (6)	98 (8)	111 (5)
H7CDD	250	116 (6)	105 (7)	106 (5)	126 (13)	109 (20)	94 (34)	99 (6)	115 (5)
08CDD	375	115 (33)	124 (18)	99 (13)	119 (13)	123 (17)	80 (53)	104 (21)	114 (10)

(1) Mean surrogate recovery (%) (n=3)
(2) CV% = $Sx/\bar{X}.100$
(3) Gross interference present

3.2 Method #8's Performance Using 3 Spike Levels:

The sample size was increased to 25 g for the following experiments. The data from the 100 ppt spike experiment (Table 3) indicated that both recoveries (98% - 105%) and precision (9% - 12%) were acceptable for each surrogate.

Recoveries (89% - 100%) and precision (6% - 17%) were also acceptable for each surrogate in the 20 ppt spike experiment (Table 4). Apart from losses of $^{13}C\text{-}O_8CDD$ in samples 1 and 2, no obvious deterioration in method performance was apparent. Native PCDD/PCDF congeners were also determined with acceptable precision (11% - 17%). The surrogate recoveries were also acceptable in the 10 ppt experiment (Table 5); although, in this case, decreased

^{13}C-P_5CDD - ^{13}C-H_7CDD congener recoveries, without associated loss of precision, were observed. The data for the native congeners were acceptably precise (7% - 14%). The levels of native congeners and homologues were similar to those obtained using Method #1 (n=3): 2,3,7,8-TCDD = 34 pg/g; 2,3,7,8-TCDF = 37 pg/g; P_5CDD = 8 pg/g; H_6CDF = 10 pg/g.

TABLE 3. METHOD PERFORMANCE USING HIGH LEVEL SURROGATE SPIKE

13C12 CONGENER	LEVEL (pg/g)	REPLICATE 1	2	3	4	5	6	7	8	$\bar{X}$(1)	CV%(2)
T4CDD	100	94	99	114	82	95	107	90	100	98	10
P5CDD	200	102	92	116	86	101	110	93	106	101	10
H6CDD	200	102	90	118	91	107	108	97	101	102	9
H7CDD	200	109	94	129	97	112	112	94	95	105	12
08CDD	300	99	88	112	98	116	108	93	80	99	12

(1) Mean surrogate recovery (%)
(2) CV% = Sx/$\bar{X}$.100

TABLE 4. METHOD PERFORMANCE USING INTERMEDIATE LEVEL SURROGATE SPIKE

CONGENER	LEVEL (pg/g)	REPLICATE 1	2	3	4	5	6	7	$\bar{X}$(1)	CV%(2)
		RECOVERY (%)								
13C12-T4CDD	20	94	84	98	94	87	91	101	93	6
13C12-P5CDD	40	78	95	83	100	83	87	96	89	10
13C12-H6CDD	40	85	93	101	114	90	83	104	96	11
13C12-H7CDD	40	116	92	108	102	97	82	103	100	11
13C12-08CDD	60	80	80	99	116	121	89	111	99	17
		CORRECTED(3) CONCENTRATION (pg/g)								
2,3,7,8-TCDD		36	47	33	48	35	37	34	38	16
2,3,7,8-TCDF		37	53	39	41	38	34	34	39	17
P5CDF(4)		7	9	9	9	11	8	9	9	13
H6CDF(4)		8	11	9	11	11	11	10	10	11

(1) Mean surrogate recovery (%)
(2) CV% = Sx/$\bar{X}$.100
(3) Data corrected for surrogate losses
(4) Total homologues

TABLE 5. METHOD PERFORMANCE USING LOW LEVEL SURROGATE SPIKE

CONGENER	LEVEL (pg/g)	REPLICATE 1	2	3	4	5	6	7	8	CV%[2]	$\bar{X}$[1]
		RECOVERY (%)									
13C12-T4CDD	10	81	85	100	105	97	115	97	87	12	96
13C12-P5CDD	20	81	86	98	91	83	79	81	87	7	86
13C12-H6CDD	20	92	106	103	91	78	77	94	85	11	91
13C12-H7CDD	20	104	97	92	84	80	81	105	87	11	91
13C12-08CDD	30	107	115	117	94	115	93	102	100	9	105
		CORRECTED[3] CONCENTRATION (pg/g)									
2,3,7,8-TCDD		34	34	32	29	35	25	31	31	10	31
2,3,7,8-TCDF		36	23	39	37	36	31	38	34	15	34
P5CDF[4]		8	8	9	8	8	9	8	8	7	8
H6CDF[4]		11	10	10	10	10	13	12	14	14	11

(1) Mean surrogate recovery (%)
(2) CV% = Sx/$\bar{X}$.100
(3) Data corrected for surrogate losses
(4) Total homologues

TABLE 6. METHOD PERFORMANCE USING A VARIETY OF FISH SAMPLES [c]

13C12 CONGENER	LEVEL (pg/g)	FISH SAMPLE (LAB. #) CARP (JH1)	CARP (JH2)	L. TROUT (JH3)	CARP (JH4)	L. TROUT (JH5)	L. TROUT (JH6)	L. TROUT (JH7)	L. TROUT (JH8)	WALLEYE (JH9)	CARP (JH10)	$\bar{X}$[2]	CV%[3]
T4CDD	50	99[1]	94	86	94	75	109	113	118	95	127	101	16
P5CDD	100	78	78	93	90	73	80	78	90	87	94	84	9
H6CDD	100	67	79	69	102	81	63	56	86	80	121	80	24
H7CDD	100	78	100	89	89	80	72	74	89	82	99	85	1
08CDD	200	67	109	129	83	96	78	101	88	82	103	94	19
Lipid Content (%)		9.8	10.7	12	11	13.6	16.3	16.5	19.6	10	9		

(1) Surrogate recovery (%)
(2) Mean surrogate recovery (%)
(3) CV% = Sx/$\bar{X}$.100

3.3 Method Performance Using a Variety of Fish Samples:

Method #8 was evaluated using 10 fish samples (25 g) (Table 6). Blockages of the GPC unit occurred during chromatography of JH1, JH6, JH7, and JH8; some extract losses occurred. High lipid extracts should probably not be allowed to remain in the sample loops longer than necessary, so as to reduce the possibility of line blockage. Despite this problem, surrogate recoveries were generally acceptable, and would probably improve with sample replication. In only one case (JH5) was the ^{13}C-T_4CDD recovery less than 80%. Some ^{13}C-O_8CDD recoveries were lower than the corresponding ^{13}C-T_4CDD values. Although recoveries tended to be lower for the ^{13}C-P_5CDD - ^{13}C-H_7CDD surrogates, in no case was an uncorrectable loss incurred.

4.0 DISCUSSION

Method #1 (Afghan et al., 1987), was modified for the present study by increasing the extraction acid's strength from 1 - 6 M, and incorporating an automated GPC step. GPC automation boosted sample throughput from 1.5 samples to 8 samples per day. Emulsion problems associated with Method #1's liquid phase steps do not, in our experience, facilitate the analysis of large (> 10g) fish samples. Solid phase procedures, are free of such problems. The presented data, while of a preliminary nature, verify that solid phase extraction and clean-up can improve ana-

lytical efficiency and effectiveness.

Solid phase extraction, which has been used by Norstrom (1986) and Smith et al. (1984), among others, is efficient. It facilitates the simultaneous and efficient extraction of multiple samples: a single operator can extract 16 samples a day. Whereas, liquid phase extraction permits the simultaneous extraction of about 6 samples by a lone operator. Furthermore, solid phase extraction has fewer equipment and glassware requirements.

The elimination of either the acid/base treatments or the alumina chromatography step compromised analytical performance. Elimination of the former was associated with erratic surrogate recoveries, possibly caused by interference of residual lipids or biogenic molecules with the alumina or carbon fibre chromatography. Such inconsistent performance would probably worsen with larger samples. Similar considerations apply to the elimination of the alumina chromatography step; in this case, the gross interferences that co-chromatographed with ^{13}C-2,3,7,8-TCDD were probably PCBs.

The multi-layer clean-up column, which is based on the "DOW" clean-up strategy (Lamparski and Nestrick, 1980) offers several practical advantages. Large samples can be processed without encountering emulsion associated problems. Multiple samples can be processed simultaneously, 20 per day being a rea-

sonable number for a single operator. In contrast, it takes a single operator about 2 days to process 8 samples using liquid phase acid/base treatments. Material costs are also lower for the solid phase method.

The correction of native PCDDs/PCDFs for surrogate losses did not consistently improve analytical precision, although it tended to increase analyte estimates in the low and intermediate spike experiments. The failure to improve analytical precision for the PCDF parameters may result from the use of the corresponding PCDD surrogate to correct for method losses. However, the reason for the lack of improvement in the precision of the corrected 2,3,7,8-TCDD data is not clear. Correlation analysis failed to detect a consistent relationship between the surrogate and native 2,3,7,8-TCDD recoveries ($P>0.05$).

Further validation of Method #8's analytical precision, using fish matrices that contain a variety of congener profiles, is required: some of the fish listed in Table 6 would be suitable. Participation in round robin studies would enable conclusions to be drawn regarding the comparative performances of Method #8 and other methods, while also providing information on analytical accuracy. Method #8 should be readily adaptable for use with other matrices, although validation would be required in each case.

5.0 REFERENCES

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