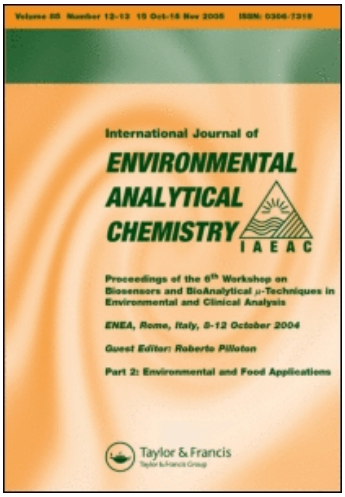

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Method for the Determination of Three Toxic Non-*Ortho*chlorine Substituted Coplanar PCBs in Environmental Samples at Part-per-Trillion Levels

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A method has been devised combining alkali digestion, carbon chromatography and high-resolution gas chromatography for the determination of 3,3′,4,4′-tetra, 3,3′,4,4′,5-penta, and 3,3′,4,4′,5,5′-hexachlorobiphenyl, the biologically active congeners of PCBs and approximate isostereomers of 2,3,7,8-TCDD. This method permits determinations of parts-per-trillion levels of these toxic residues in biological samples. Interference both from biogenic and from xenobiotic substances was reduced to extremely low levels. Using this method, 13.5 ng of 3,3′,4,4′-tetrachlorobiphenyl/g, 0.89 ng of 3,3′,4,4′,5-pentachlorobiphenyl/g and 0.64 ng of 3,3′,4,4′,5,5′-hexachlorobiphenyl/g were detected on wet weight basis in the blubber of a finless porpoise. To our knowledge this is the first report on the three toxic non-*ortho* chlorine substituted PCB residues detected in a higher mammal in the wilderness.

KEY WORDS: Coplanar PCBs, non-*ortho* chlorine substituted PCBs PCDDs, carbon chromatography, alkali digestion, high-resolution gas chromatography.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are highly stable and lipophilic

industrial pollutants whose residues are found in many components of the global ecosystem.[1–3] Increasing evidences suggest that a large portion of PCB toxicity may be associated with small amounts of non-*ortho* chlorobiphenyls having 4 or more chlorine atoms in vicinal *meta* and *para* ring positions (exhibiting maximum coplanar conformational character).[4,5] The most active of them viz. 3,3′,4,4′-tetrachlorobiphenyl(TeCB), 3,3′,4,4′-5-pentachlorobiphenyl(PeCB) and 3,3′,4,4′,5,5′-hexachlorobiphenyl(HeCB) elicit toxic responses typical of the highly toxic 2,3,7,8-TCDD, including a wasting syndrome, thymic atrophy, hepatic damage, reproductive toxicity, differential effects on genetically inbred C57BL/6J and DBA/2J mice, porphyria, immunotoxicity and dermal toxicity and resemble 3-methylcholanthrene (3-MC) in their mode of induction of hepatic drug-metabolising enzymes.[6] Although long-term toxicity studies have not been reported so far for these PCB congeners; it can be presumed that they are one of the real threats to the wildlife and humans alike.

The extreme toxic potential of these non-*ortho* chlorine substituted PCBs when considered in the light of limited knowledge about their occurrence and distribution in the environment, necessitates the development of isomer specific analytical method for this group of compounds.

Jensen and Sundström[7] initially used an active carbon for the fractionation of PCBs according to their degree of *o-o′*chlorine substitution. Carbon has a high affinity for certain planar aromatic compounds—especially those with adjacent aromatic rings and increasing electronegative substitutes in the planar aromatic rings.[8] There are several reports using carbon chromatography fractionating the planar compounds such as polychlorinated dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs) and *o-o′* chlorine unsubstituted PCBs and naphthalenes from other classes of aromatic compounds.[8–13] The common features of all these techniques involve several additional chromatographic separations including HPLC (GPC), silica gel, potassium silicate and alumina, either before or after carbon chromatography which are complicate and time consuming. Hence we attempted to develop a method which was simple, sensitive, rapid and specific for non-*ortho* chlorine substituted 3,3′,4,4′-TeCB, 3,3′,4,4′,5-PeCB and 3,3′,4,4′,5,5′-HeCB for routine laboratory analysis of these compounds.

EXPERIMENTAL SECTION

The analysis of TeCB, PeCB and HeCB essentially involved the following steps (Figure 1): (1) extraction and saponification of lipids using alkaline ethanol, (2) cleanup and fractionation using activated charcoal, (3) an additional cleanup using fuming sulfuric acid, (4) isomer specific determinations using HR-GC-ECD and, (5) confirmation with GC-MS.

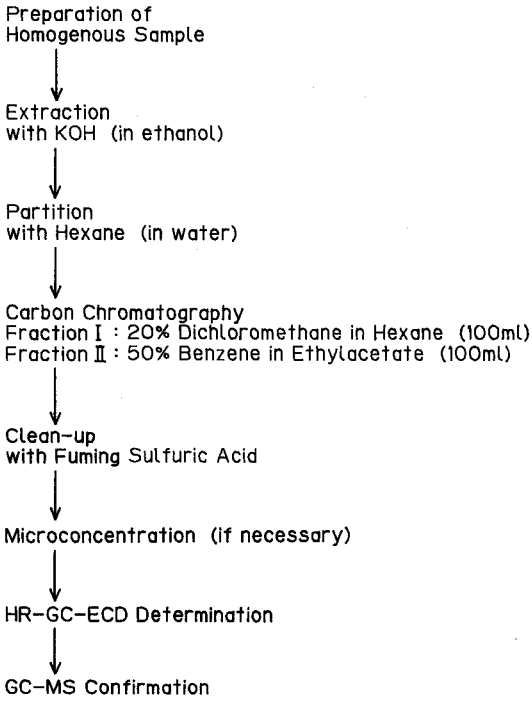

Figure 1 Flow chart of total procedure.

Sample preparation and extraction

Ten to 20 g of biological fat sample (blubber of a marine mammal, in this case) was cut into small pieces and digested using 300 to 500 ml of 1N KOH in ethanol for one hour. After cooling down to *ca* 50°C,

this extract was transferred to a separating funnel containing equal volume of water (hexane-washed) as that of the alkaline ethanol and 100 ml of hexane. After thorough partitioning the hexane phase was collected carefully and concentrated to 5 ml in a Kuderna–Danish concentrator.

Cleanup and fractionation in carbon chromatography

The active carbon from Wako Pure Chemical Industries Ltd., Japan was used as the adsorbent without any further activation. Chromatographic columns were 5 mm i.d. glass filled to 35 mm bed depth with carbon (125 mg) using hexane. A desired volume of hexane extract from the first step was added to this column and eluted initially with 100 ml of 20% dichloromethane in hexane at one drop per second flow rate. This fraction was discarded and elution was continued with 50% benzene in ethyl acetate. This second fraction was concentrated using a Kuderna–Danish concentrator.

Sulfuric acid cleanup

It was further concentrated carefully under nitrogen atmosphere to 100 μl, to be made up to 5 ml volume using distilled hexane. 5 ml of 5% fuming sulfuric acid in concentrated sulfuric acid was added to this and after shaking the upper hexane layer was collected carefully and washed with 5 ml hexane-washed water.

High-resolution GC-ECD analysis

An aliquot from the hexane extract was injected into Shimadzu GC-9A model gas chromatograph fitted with 0.25 mm i.d. $\times$ 25 m fused silica capillary column with chemically bonded OV-1701 and equipped with ^{63}Ni EC detector, using a moving needle type injection system (splitless and solvent cut mode). The oven temperature was programed at a rate of 0.2°C/min from 220° to 240°C. The injector and detector temperatures were 250°C. The carrier gas nitrogen was maintained at 1.5 kg/cm^2 pressure. At these GC conditions TeCB, PeCB and HeCB were eluted at 14.9 min, 24.4 min and 38.7 min respectively. The sample peaks were calibrated against the standard peaks recorded in a Shimadzu CR2AX printer plotter.

Further confirmation of these non-*ortho* PCBs was obtained using WCOT silicone OV-101 glass capillary column (0.23 mm i.d. × 30 m) fitted to Shimadzu GC-7A equipped with EC detector and SIC chromatocorder 11 model integrator.

GC-MS confirmation

Mass fragmentographic data were obtained with Shimadzu model GC-MS-QP 1000 operated in the electron impact mode and interfaced with a Shimadzu GC-9A equipped with a moving needle type injection system. The chemically bonded OV-1701 fused silica column (0.25 mm i.d. × 25 m) was used. The temperature program was as follows: the initial column temperature was held at 220°C for 5 min after injection and increased at 0.2°C/min to 240°C. The injector temperature was 240°C, separator, 240°C, ion source, 250°C. The carrier gas was helium with a flow rate of 1.2 ml/min. The accelerating voltage was +3.5 KV, trap current was 60 μA and the ion energy was 70 eV.

In this study of non-*ortho* chlorine substituted PCBs, the masses and known relative abundances of M^+ and $(M+2)^+$ cluster ions were used as the identification and quantification criteria (for example *m/z* 290 and 292 for TeCB, *m/z* 324 and 326 for PeCB and *m/z* 358 and 360 for HeCB). The ratio of the confirmation ion intensity to the quantitation ion intensity was calculated and compared with the expected ratio for each level of chlorination.

Materials

All solvents were glass distilled grade. Carbon (charcoal) used in this study was of the following particle size: coarser (297 μ) <40%; 297 μ–63 μ >50%; finer than 63 μ <10%; loss on drying (at 105°C) <15% and the lot number was CDE 7469. The KOH and sodium sulfate (anhydrous) were of analytical grade. The adsorbent carbon, all the solvents and glass wares used in this study were devoid of any non-*ortho* PCB contamination.

3,3',4,4'-TeCB, 3.3',4,4',5-PeCB, and 3,3',4,4',5,5'-HeCB (IUPAC No. 77, 126 and 169) were originally synthesized and identified at the following institutes: 3,3',4,4'-TeCB and 3,3',4,4',5,5'-HeCB at Osaka Prefectural Institute of Public Health, Osaka, Japan and 3,3',4,4',5-PeCB

at the National Institute for Environmental Studies, Tsukuba, Japan. Further checking of the purity after their arrival to our laboratories using HR-GC-ECD and HR-GC-FID revealed a purity level of 98–100%.

RESULTS AND DISCUSSION

Development and functions of the components of the analytical procedure

During alkali digestion the non-*ortho* chlorine substituted PCBs were extracted in ethanol and the lipid, bio-organic materials and organophosphorous pesticides were digested in alkali. The lipid thus saponified was eliminated later along with the water phase in the hexane partitioning step. Thus the major cleanup was achieved in the digestion step itself. This method has been thoroughly standardized in this laboratory and is being used routinely for the analysis of PCBs in environmental samples.[14]

The activated carbon was found to separate chlorinated aromatics on the basis of molecular planarity (PCB planarity is governed primarily by *o-o'* chlorine substitution) and degree of chlorination[10] and hence superior to florisil or silica gel chromatography, which are based primarily on solute polarity. The carbon from Wako Pure Chemical Industries Ltd., Japan was easy to pack without giving any back-pressure because of its coarseness. Thus the polyurethane and glass fibre carriers used in the previous methods were avoided.[10,11] While PCBs with decreasing number of chlorines in the *ortho* positions are selectively retained in the carbon column, all chlorinated pesticides, PCBs with at least one *ortho* substituent are eluted in the first 100 ml of 20% dichloromethane in hexane (Figure 2). It has been thoroughly checked that none of these compounds appear in any significant level in the second fraction of 100 ml 50% benzene in ethyl acetate. Additionally a considerable portion of interfering biogenic materials is eliminated in the first fraction. TeCB, PeCB and HeCB appear mainly in the second fraction. Spiking with PCDDs and PCDFs indicated their absence in this fraction. An elution with a strong solvent such as toluene was needed to elute those compounds. Thus the present elution procedure for the carbon chromatography saves both time and

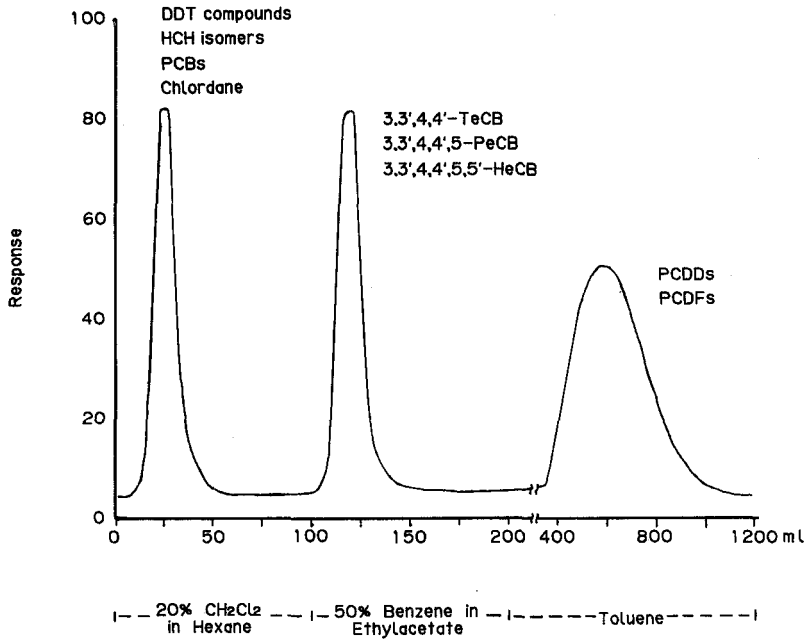

Figure 2 A schematic elution profile of organochlorine compounds in carbon chromatographic method.

materials as compared to the elution with incremental gradient of toluene in cyclohexane using more than a litre of solvents.[15]

The high resolving power of chemically bonded capillary GC columns was additionally utilized to separate the non-*ortho* chlorine substituted PCBs of interest from other organochlorine compounds. Test revealed that with the prescribed GC conditions using OV-1701 capillary column, TeCB, PeCB and HeCB could be determined without the interference of either HCH isomers, DDT compounds, PCDDs, PCDFs or Chlordane.

Recovery studies were undertaken to check the efficiency of the charcoal column as well as the total procedure. Initially charcoal column was tested. A standard mixture of 23.4 ng TeCB/ml, 10.0 ng PeCB/ml and 15.2 ng HeCB/ml was prepared and subsequent dilution to 1 and 2 order less yielded the other two spiking solutions. 5 ml from each of these standard mixtures was spiked in triplicate on the charcoal column and eluted. The second fraction of 50% benzene

Table I Recoveries of 3,3′,4,4′-tetrachlorobiphenyl (TeCB), 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) and 3,3′,4,4′,5,5′-hexachlorobiphenyl (HeCB) in Carbon Column (A), Total Procedure (without blubber) (B) and Total Procedure (with blubber) (C).

Level of spiking		Coplanar PCBs	Amount added (ng/g blubber)	Amount added (ng)	Found(ng)	Recovery (%)
A	I	TeCB		117	109±3.7	93±3
		PeCB		50.0	47.1±1.7	94±3
		HeCB		76.0	64.8±3.0	85±4
	II	TeCB		11.7	11.5±0.35	98±3
		PeCB		5.00	4.94±0.22	99±4
		HeCB		7.60	6.13±0.19	81±3
	III	TeCB		1.17	0.90±0.05	77±4
		PeCB		0.50	0.43±0.02	86±5
		HeCB		0.76	0.60±0.01	79±2
B	I	TeCB		117	106±5.3	91±4
		PeCB		50.0	44.7±2.6	89±4
		HeCB		76.0	51.7±2.5	68±3
	II	TeCB		11.7	10.2±0.05	87±1
		PeCB		5.00	4.53±0.16	91±3
		HeCB		7.60	5.58±0.09	73±1
	III	TeCB		1.17	0.95±0.06	81±5
		PeCB		0.50	0.42±0.01	84±2
		HeCB		0.76	0.55±0.01	72±1
C	I	TeCB	11.7	117	111±2.2	95±2
		PeCB	5.00	50.0	45.0±2.0	90±4
		HeCB	7.60	76.0	53.0±0.8	70±1
	II	TeCB	1.17	11.7	8.30±0.82	71±7
		PeCB	0.50	5.00	3.60±0.22	72±4
		HeCB	0.76	7.60	5.00±0.16	66±2
	III	TeCB	0.117	1.17	0.67±0.04	57±4
		PeCB	0.050	0.50	0.30±0.05	60±10
		HeCB	0.076	0.76	0.42±0.02	55±3

Notes: Recoveries are from triplicate analyses with mean and standard deviation.

in ethyl acetate was collected, micro-concentrated, and made up to 5 ml with hexane, cleaned up with sulfuric acid and determined using HR-GC-ECD. There was no degradation of TeCB, PeCB and HeCB by fuming sulfuric acid. The recovery was found to be satisfactory with good reproducibility (Table I). The recovery for TeCB and

PeCB was generally more than 90% and for HeCB, it was between 80–85%. Thus, this carbon chromatographic method yielded higher recoveries for these non-*ortho* chlorine substituted PCBs when compared to the method of Huckins *et al.*[10] with carbon column and Kamops *et al.*[16] with florisil column.

Five millilitres from each of the standard mixtures mentioned earlier was spiked in triplicate on a 10 g blubber sample of minke whale (*Balaenoptera acutorostrata*). This specimen was caught in the pristine environment of Antarctic and the total PCB concentration was in the lower ppb levels. A blank check of this blubber revealed undetectable levels of TeCB, PeCB and HeCB with no interference from matrix (fat) residues from 10 g of the sample. Concurrently, spiking was done at similar dose levels without minke whale blubber. Results are summarised in Table I.

Spiking blubber at level I; 12 ppb-TeCB, 5 ppb-PeCB and 8 ppb-HeCB, yielded excellent recoveries. This was true for spiking without blubber, too. Spiking at the levels II and III which were in the higher and medium ppt ranges (for blubber) resulted in lower recoveries (which was still above 60% on an average). However, the recoveries of spiking experiments without blubber were good and comparable to carbon chromatographic recoveries. With 100 ppb spiking, Smith *et al.*[12] recovered 38 and 57% of TeCB, 43 and 47% of PeCB and 54 and 59% of HeCB using their procedure. In general, all the available methods on non-*ortho* chlorine substituted PCBs used high spiking concentrations (microgram or sub-microgram) whereas the proposed method of ours yielded good recoveries even at low levels of spiking.

An adult male finless porpoise (*Neophocoena phocoenoides*) (162.4 cm body length) was found dead on the shores of Matsuyama, Japan on July 17, 1985. The total PCBs in the subcutaneous blubber was found to be 39.8 μg/g on a wet weight basis. Further analysis of its blubber using this method revealed the presence of all the three toxic congeners (Figures 3 and 4). In the GC-ECD method these congeners were separated, identified and determined using OV-1701 and OV-101 capillary columns. The determined concentrations using OV-1701 were 13.5 ng TeCB/g, 0.89 ng PeCB/g and 0.64 ng HeCB/g. This was in close agreement with determinations using OV-101 capillary column in a different temperature program (Table II). The lowest detectable level of these three non-*ortho* chlorine substituted PCBs in blubber sample by GC-ECD in our study was 25 pg/g.

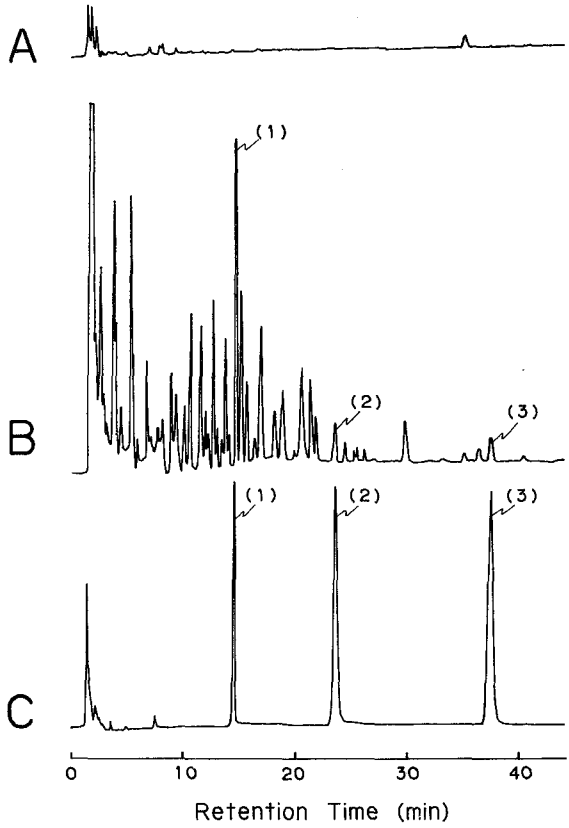

Figure 3 OV-1701 HR-GC-ECD analysis of finless porpoise for coplanar PCBs. A. blank, B. finless porpoise blubber, and C. standard. Chromatograms A and B were obtained by an injection of 20 μl hexane extract. C was obtained by injecting 5 μl of a standard mixture containing (1) 117 pg TeCB, (2) 50 pg PeCB and (3) 76 pg HeCB.

Additional confirmation was made using mass fragmentography on M^+ and $(M+2)^+$ cluster ions of non-*ortho* PCBs (Figure 4). The quantified values based on these ions agreed well with the results of GC-ECD (Table II). Moreover, the ratios of intensities of both cluster ions resulting from expected natural abundance of ^{35}Cl and ^{37}Cl showed similar values in standards and finless porpoise (Table III). These observations confirmed the presence of TeCB, PeCB and HeCB in finless porpoise. Interestingly, GC-MS analysis

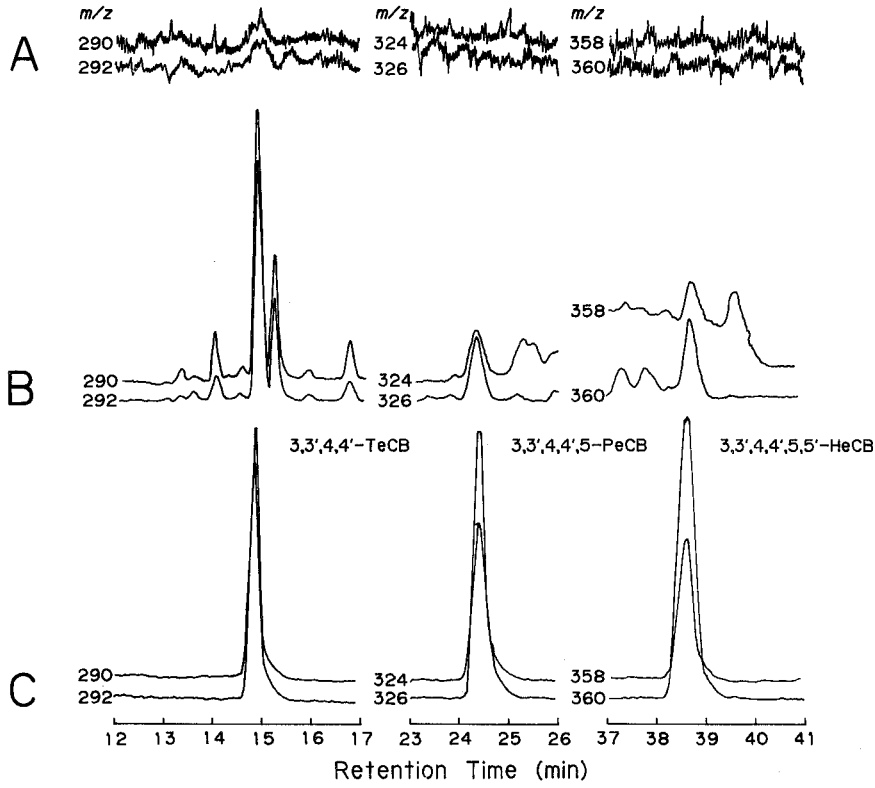

Figure 4 Mass fragmentographic analysis of coplanar PCBs in procedural blank (A), finless porpoise (B) and standard (C). Fragmentogram C was obtained by an injection of 5 μl of standard mixture (TeCB = 230 pg, PeCB = 230 pg, HeCB = 400 pg). 10 μl of a micro-concentrated hexane extract of blubber of finless porpoise was injected to obtain B. The blank was fragmentogramed at 100 times higher sensitivity.

revealed the probable presence of other coplanar congeners (especially tetra) in finless porpoise (Figure 4). However, due to unavailability of technical standards, those congeners were not analysed. The extra peaks along with the confirmed non-*ortho* chlorine substituted PCBs in Figure 3 might also be due to compounds with planar configuration of unknown origin. We are interested in understanding the nature of these compounds as their biological implications are unknown at present.

Table II Concentrations of non-*o*,*o*'-Cl substituted PCB congeners in finless porpoise from Seto-Inland Sea, Japan.

Coplanar congeners	Concentration (ng/g) on wet weight basis				
	HR-GC-ECD		GC-MS (bonded OV-1701)		
	Bonded OV-1701	WCOT OV-101	Quantitation ions	Residue ng/g	Mean
3,3',4,4'-TeCB	13.5	17.0	290 292	16.2 16.2	16.2
3,3',4,4',5-PeCB	0.89	1.30	324 326	1.23 1.18	1.20
3,3',4,4',5,5'-HeCB	0.64	0.57	358 360	0.70 0.72	0.71

Table III Mass fragmentographic analysis of abundance ratios of coplanar PCBs in finless porpoise.

Coplanar PCBs	*m/z*	Abundance ratio in technical standard	Abundance ratio in finless porpoise
TeCB	290:292	1.00:1.26	1.00:1.26
PeCB	324:326	1.00:1.70	1.00:1.63
HeCB	358:360	1.00:1.95	1.00:2.01

Our observations indicate that carbon chromatography in combination with HR-GC-ECD offers an easy method for routine analysis. However, the determination of coplanar PCBs depends on the level of co-contaminants whose level seems to vary according to the tissue of the animal species. Thus, confirmation by GC-MS is necessary, if there are several interfering peaks in the chromatogram.

Significance of coplanar PCBs in environmental sample

To our knowledge this is the first report on the occurrence of three toxic non-*ortho* chlorine substituted PCB residues in a higher mammalian species in the wilderness. The other reports viz. 10.2 ng 3,3',4,4'-TeCB/g in fish from Hudson River[9] and 0.11 μg 3,3',4,4'-TeCB/g in carp caught in Ohio River,[10] are from lower vertebrates and

reported only tetrachlorobiphenyls. *Ortho* unsubstituted PCBs (especially TeCB, PeCB and HeCB) were reported at high levels in Forster's Tern eggs in a recent report to U.S. Fish and Wildlife Service.[17] All three reports utilized HR-GC-ECD and Mass Fragmentographic confirmations.

3,3',4,4'-TeCB, 3,3',4,4',5-PeCB and 3,3',4,4',5,5'-HeCB are biologically most active and comparatively the most toxic of the PCB congeners tested.[18,19] They are found to be potent inducers of hepatic drug-metabolizing enzymes,[20,21] immunotoxic,[22] teratogenic, embryotoxic[23,24] and inhibiting steroid metabolism[25] in experimental animals. At least the more chlorinated and *para* substituted 3,3',4,4',5-PeCB and 3,3',4,4',5,5'-HeCB are likely to persist in the environment and living organisms. Their long-term effects to humans and wild animals is not known at present. TeCB, PeCB and HeCB are approximate isostereomers of the highly toxic 2,3,7,8-TCDD. The induction of ethoxyresorufin *O*-deethylase and benzo(a)pyrene hydroxylase in rat hepatoma cell cultures by 3,3',4,4',5-PeCB was only 3- to 4-fold lower than the data obtained for the highly toxic 2,3,7,8-TCDD.[20] The 3,3',4,4',5,5'-HeCB and 3,3',4,4'-TeCB are only 30 and 80 times less toxic (LD_{50}) to the highly toxic 2,3,7,8-TCDF.[26] So far the reported levels of 2,3,7,8-TCDD and 2,3,7,8-TCDF in environmental samples have been in parts-per-trillion levels[27–31] whereas the TeCB, PeCB and HeCB are found in parts-per-billion and are expected in the same range. Thus the possibility of TeCB, PeCB and HeCB with high toxic potency accumulating in animals at a higher level than 2,3,7,8-TCDD and 2,3,7,8-TCDF warrants an immediate and critical evaluation of the problem. There is an urgent necessity now, to understand their occurrence and distribution in the wild animals and human populations.

The present analytical method which is sensitive and rapid is developed to cope with this necessity of monitoring these toxic residues in environmental samples.

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