# Polychlorinated hydroxybornanes - metabolites of toxaphene in livers of polar bears (*Ursus maritimus*)

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## Introduction

Toxaphene was one of the most heavily used non-systemic organochlorine pesticides worldwide with a production rate of ~1.3 million tons.[1] The chloropesticide was produced by the chlorination of α-pinene/camphene,[2] which results in a complex mixture of >1,000 of more than 30,000 possible compounds of technical toxaphene (CTTs).[3,4] Production and use of toxaphene were stopped in most countries by its classification as persistent organic pollutant (POP) by the Stockholm Convention in 2004. However, toxaphene is still present in environmental samples, and especially in fatty fish and marine mammals.[5-7] Typically, the toxaphene residue pattern in higher organisms was much less complex than the technical product.[8] For instance, one octa- and one nonachlorobornane - 2-*exo*,3-*endo*,5-*exo*,6-*endo*,8,8,10,10-octachlorobornane (B8-1413)[9] and 2-*exo*,3-*endo*,5-*exo*,6-*endo*,8,8,9,10,10-nonachlorobornane (B9-1679)[9] - were found to be particularly recalcitrant in marine mammals.[10,11] Transformation of the many hundred less persistent CTTs may occur abiotically by UV light.[12] Compared to that little is known about the mechanisms of biotic transformation of toxaphene.[13-16] Next to reductive dechlorination,[8] hydroxylation via the phase-I-reaction of the detoxification metabolism represents one plausible degradation pathway. Scarcely, hydroxylated toxaphene metabolites were observed under laboratory conditions.[11-13] However, hydroxylated CTTs (OH-CTTs) have not been detected so far in authentic environmental samples.[17]

The aim of this study was to develop a method for the verification of the occurrence of OH-CTTs in environmental samples. For this purpose, an OH-CTT mixture was synthesized [17] and fractionated with countercurrent chromatography (CCC). CCC is an all liquid based (semi-) preparative instrumental chromatography method typically used for the isolation of natural products [18], which was previously also successfully used for the fractionation of toxaphene [19]. The resulting, less complex OH-CTT mixture consisting of only ~20 OH-CTTs was used to develop a sample clean-up method which provided a full separation of both matrix compounds and isobaric PCBs which show the same molecular ion and hence would interfere the analysis by gas chromatography with electron-capture negative ion (low resolution) mass spectrometry (GC/ECNI-MS). This method was finally applied to analyze livers of polar bears.

## Materials and methods

**Chemicals and standards**. L-bornyl acetate was from Merck (Darmstadt, Germany) and *n*-hexane (HPLC grade, ≥95.0%; residue analysis grade, ≥99.0%), cyclohexane (≥99.5%) and ethyl acetate (≥99.5%, distilled prior to use) were from Th. Geyer (Renningen, Germany). Pyridine (for HPLC, ≥99.9%, distilled prior to use), *iso*-octane (for pesticide residue analysis), toluene (for pesticide residue analysis), cholesterol (>99.0%), sulfuryl chloride (≥97.0%) and silica gel 60 were from Sigma Aldrich (Steinheim/Seelze, Germany). Acetonitrile (gradient grade for HPLC, ≥99.9%) and methanol (gradient grade for HPLC, ≥99.0%) were from VWR chemicals (Darmstadt, Germany), whereas *n*-butanol (≥99.0%) and sodium sulfate (water-free, *p.a.*, ≥99.0%) were both from Carl Roth (Karlsruhe, Germany). Demineralized water was produced in-house (ELGA PURELAB system, Celle, Germany). A mix of the PCB congeners (Dr. Ehrenstorfer, Augsburg, Germany) was prepared in *iso*-octane (PCB-mix). Perdeuterated α-HCH (α-PDHCH) and 6'-MeO-BDE 66 (BCIS) were used as internal standards.

**Samples**. Fish oil capsules "Omega-3 1000" (tetesept Pharma, Frankfurt a. M., Germany) were used as fish oil simulation matrix for development of the clean-up procedure. For this purpose, 500 mg fish oil was spiked with 150 µL PCB-mix only, 150 µL OH-CTT-mix (CCC fraction F27, 65.9 min, see below) only, and with both mixtures respectively. Furthermore, clean-up of liver samples of four polar bears (*Ursus maritimus*) from Iceland has been described in detail in a previous study [18]. GC/ECNI-MS analysis of all four polar bear livers showed octachloro-CTTs at 65-320 µg/kg lipid weight. PCBs were about 100 fold more abundant in the polar bears livers.

**Synthesis of OH-CTTs**. L-bornyl acetate (2.0 g in 30 mL sulfuryl chloride) was photochlorinated with a water-cooled TQ150 medium pressure mercury vapor UV lamp (150 W, Heraeus/Peschl Noblelight, Hanau/Germany) according to Kapp and Vetter [17]. A 25 mL-aliquot was taken after 24 h irradiation. Further clean-up steps, performed according to Kapp and Vetter [17], resulted in 1.6 g OH-CTT synthesis feedstock.

**Countercurrent chromatography (CCC)**. CCC fractionation of 790 mg OH-CTT synthesis product was performed with an AECS Quikprep MK8 instrument (AECS, London, United Kingdom) using bobbin 1 (stainless steel tubing, column volume 238 mL, 2.1 mm i.d.) and a 10 mL sample loop. Rotation speed was set at 860 rpm and the temperature was kept at 25 °C. The solvent system (*n*-hexane, ethyl acetate, water, methanol,

40:10:40:10, *v/v/v/v* [21]) was used in tail-to-head mode. The solvent was pumped by a ternary Beta 50 pump (Ecom, Praha, Czech Republic) with a flow rate of 4 mL/min. Stationary phase retention ($S_f$) was 214 mL. The sample was fractionated by means of a Gilson 203B fraction collector (Middleton, WI, USA). After 169.1 min, 70 consecutive fractions of 7.2 mL were collected. Fractions were evaporated to dryness and re-dissolved in 1 mL methanol. α-PDHCH (107 ng) was added to each CCC fraction before GC/ECNI-MS analysis.

**Gas chromatography with electron-capture negative ionization mass spectrometry (GC/ECNI-MS)**. A 7890A/5975C GC/MS system (Agilent, Waldbronn, Germany) was used in combination with an HP5-MS UI column (30 m x 0.25 mm i.d., 0.25 µm $d_f$, J&W Scientific, Folsom, CA, USA). Sample solutions (1 µL) were injected via a 7693A autosampler (Agilent) into a Gerstel CIS-4 PTV injector (Mülheim, Germany) operated in pulsed splitless mode (25 psi/min, 1 min). GC oven program started for 1 min at 50 °C, then, the temperature was raised with 10 °C/min to 300 °C (4 min). The transfer line, quadrupole and ion source temperatures were set at 300 °C, 150 °C and 150 °C, respectively. He (purity 99.9990%, Westfalen, Münster, Germany) was used as carrier gas with a constant flow rate of 1.2 mL/min. Methane (purity 99.9995%, Air Liquide, Bopfingen, Germany) was used as reagent gas (2 mL/min) for measurements in GC/ECNI-MS full scan (*m/z* 50-550) and selected ion monitoring mode (SIM) mode. GC/ECNI-MS analysis of individual CCC fractions was based on the two most abundant isotope peaks of the molecular ion ($[M]^-$) of tri- to heptachlorinated OH-CTTs. Due to possible overlapping with $[M-Cl]^-$ or $[M-HCl]^-$ fragment ions of higher chlorinated isomers, it was necessary to investigate the relative isotope peak ratio of the two highest $[M]^-$ isotope peaks and to compare this to the theoretical isotope pattern (maximum deviation ±5%).

**Gel permeation chromatography (GPC)**. Samples (spiked fish oil, polar bear livers, sample blanks and CCC fraction #27) were introduced into the AccuPrep MPS system (ANTEC, Sindelsdorf, Germany) using Bio-Beads SX-3. Polyhalogenated compounds were eluted with cyclohexane/ethyl acetate (46/54, w/w) mixture (flow rate 5 mL/min) [22] into GPC fraction 2 (20 - 40 min). The solvent was changed to *iso*-octane and made up to 1 mL.

**Adsorption chromatography on deactivated silica gel (column A)**. The whole GPC extract was further purified on a 1 cm i.d. glass column filled with 1 g silica gel deactivated with 1.5wt% water which was covered with a small layer of anhydrous sodium sulfate (pre-dried for 24 h at 550 °C) using the protocol for pesticides of different polarity with modifications [23]. Fractionation of samples and initially CCC fraction #27 used for method development started with 8 mL *n*-hexane (fraction A1), followed by 8 mL *n*-hexane/toluene (65:35, v/v, A2), 16 mL toluene (A3), 8 mL toluene/acetone (99:1, v/v, A4a), 8 mL toluene/acetone (95:5, v/v, A4b) and 8 mL toluene/acetone (80:20, v/v, A5). Each fraction was concentrated to 1 mL.

**Adsorption chromatography on activated silica gel (column B)**. A 500 µL aliquot of fraction A3 (see previous step) of samples and CCC fraction #27 was fractionated on activated silica gel according to Kapp and Vetter [17]. Elution was achieved with 48 mL *n*-hexane (B1), followed by 50 mL *n*-hexane/ethyl acetate (90:10, v/v, B2) and 50 mL ethyl acetate (B3). Each fraction was evaporated and set to 500 µL *iso*-octane. After addition of 10 µL of the ISTD (200 ng BCIS), sample solutions were analyzed by GC/ECNI-MS.

## Results and discussion

**Synthesis of OH-CTTs.** Elemental analysis of the OH-CTT synthesis mixture attested an average carbon content of 22.5% (chlorine content of 75.0%) which corresponds with a mean molecular formula of $C_{10}H_{6.8}Cl_{11.2}O$. GC/ECNI-MS analysis indicated the presence of at least 148 tetra- to heptachlorinated OH-CTTs (retention time range: 17.3-23.0 min).

**CCC procedure.** The complexity of the synthesis mixture could be efficiently simplified by CCC. For instance, CCC fraction #49 only consisted of nine penta- and hexachlorinated OH-CTTs (118.7 min, Figure 2). In several occasions, isomers with the same GC retention time were detected in different CCC fractions, which indicated a higher complexity of the synthesis product than was anticipated from the inspection of the GC/ECNI-MS chromatogram. In detail, 227 different OH-CTTs were detected in the CCC fractions (12 tetra-, 117 penta-, 81 hexa- and 17 heptachlorinated OH-CTTs), which was >50% more than detected before the fractionation. Finally, CCC fraction #27 (**Figure 1**) was selected for method development.

**GC/ECNI mass spectra of PCBs and OH-CTTs**. PCBs and OH-CTTs of the same degree of chlorination are isobaric and cannot be distinguished by low resolution MS [17].

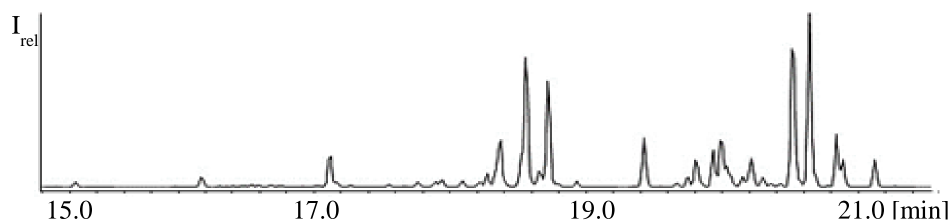


**Figure 1: OH-CTT pattern of CCC fraction #27 used for method development**

In addition, the GC retention time ranges are also virtually the same (e.g. heptachlorinated OH-CTTs: 21.1-23.0 min, heptachlorobiphenyls: 21.1.-22.5 min), and both substance classes may be mixed with each other when present in environmental samples. However, distinct differences exist in the GC/ECNI-MS fragmentation patterns of PCBs and OH-CTTs (**Figure 2**). In general, OH-CTTs fragmented stronger than PCBs. For instance, GC/ECNI-MS mass spectra of penta- to heptachlorinated OH-CTTs generally featured at least one fragment ion which was more abundant than $M^-$ while $M^-$ was generally the base peak of PCBs (**Figure 2**). Abundant GC/ECNI-MS fragment ions of OH-CTTs corresponded with loss of $Cl/Cl_2$ and/or $HCl/HCl_2$ [17] (e.g. *m/z* 357, *m/z* 321, and *m/z* 284 for heptachlorinated OH-CTTs, **Figure 2a,b**). In contrast, mass spectra of PCBs featured predominantly $[M+n*H-n*Cl]^-$ fragment ions (e.g. *m/z* 290, *m/z* 324, and *m/z* 358 in the case of heptachloro PCBs, **Figure 2d**). A further diagnostic feature of OH-CTTs was the formation of $[HCl_2]^-/[Cl_2]^-$ which were not noticed in mass spectra of PCBs. In addition, OH-CTTs showed fragment ions at *m/z* 248 ($Cl_7$-OH-CTTs), *m/z* 250 ($Cl_6$-OH-CTTs), *m/z* 252 ($Cl_5$-OH-CTTs) and *m/z* 254 ($Cl_4$-OH-CTTs) caused by elimination of four, three, two and one HCl unit, respectively, which was not observed in the corresponding GC/ECNI-MS spectra of PCBs (**Figure 2a,b** vs. **2c,d**). Hence, the final GC/ECNI-MS-SIM method was based on both isotope peaks of $M^-$ of OH-CTTs along with specific ions which enabled the unequivocal differentiation of OH-CTTs and PCBs.

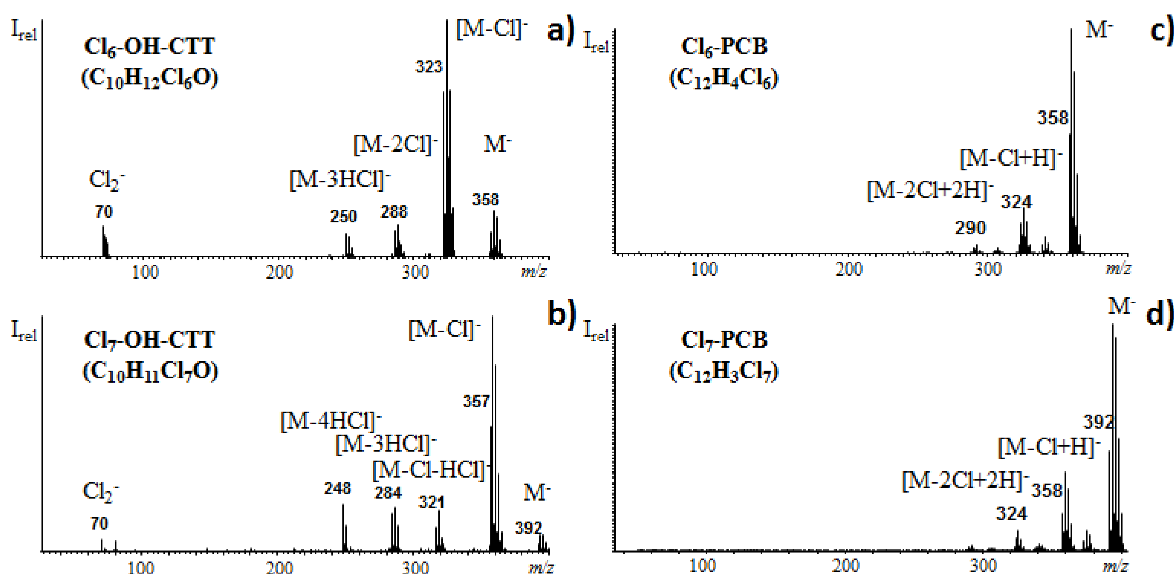


**Figure 2: GC/ECNI-MS spectra of typical OH-CCTs and PCBs with an example of a (a) hexachlorinated and (b) heptachlorinated OH-CTT as well as a (c) hexachlorinated and (d) heptachlorinated PCB**

**Removal of lipid components and PCBs**. GPC removed ~99% of the lipid matrix from the polychlorinated compounds (here: PCBs, CTTs and OH-CTTs). Yet, GPC fraction 2 still contained ~5 mg lipids, in particular cholesterol, which would lead to chromatographic interferences and matrix effects in GC/MS measurements. Column A (see Materials and Methods) allowed to fully separate OH-CTTs from cholesterol, while ~10% OH-CTTs were lost which was deemed acceptable. The bulk of PCBs (mainly eluting into fractions A1 and A2) was also separated from OH-CTTs by this step. However, fraction A3 still contained several PCBs (~6%). This share was removed on activated silica gel (column B) [17]. With this procedure PCBs were exclusively detected in fraction B1, while OH-CTTs in CCC fraction #27 eluted into fractions B2 (17%) and B3 (83%).

**OH-CTTs in polar bear liver**. Purification of four polar bear liver samples with the presented method (GPC, silica column fraction A3 and silica fraction B3) allowed the removal of all PCBs from OH-CTTs (**Figure 3a,b**). Namely, PCBs were only detected in fraction B1 whereas fraction B2 neither featured PCBs and OH-CTTs but only some unhalogenated peaks. Noteworthy, fraction B3 of all polar bear liver samples showed one abundant hexachlorinated OH-CTT (**Figure 3b**). The molecular ion at *m/z* 358 with hexachloro pattern (**Figure 3c**) was accompanied with the characteristic fragment ions corresponding to the loss of $[M-n*HCl]^-$ which were found in the synthetic OH-CTT mixture. Moreover, *m/z* 323 ($[M-HCl]^-$) was the base beak (and hence more abundant than $M^-$ ) and the mass spectrum also featured the diagnostic fragment ion at *m/z* 70 ($Cl_2^-$) (**Figure 2a,b**) which is not present in mass spectra of PCBs (**Figure 2c,d**).

Without an authentic quantification standard at hand the concentrations of the hexachlorinated OH-CTT in the polar bear liver samples was estimated by means of the GC/ECNI-MS full scan response of the isobaric PCB 153. The estimated concentrations ranged between 0.1-17 µg/kg lipid weight in the four polar bear samples. This amount was about 0.1-1.3% of the octachlorinated CTTs determined in these livers.

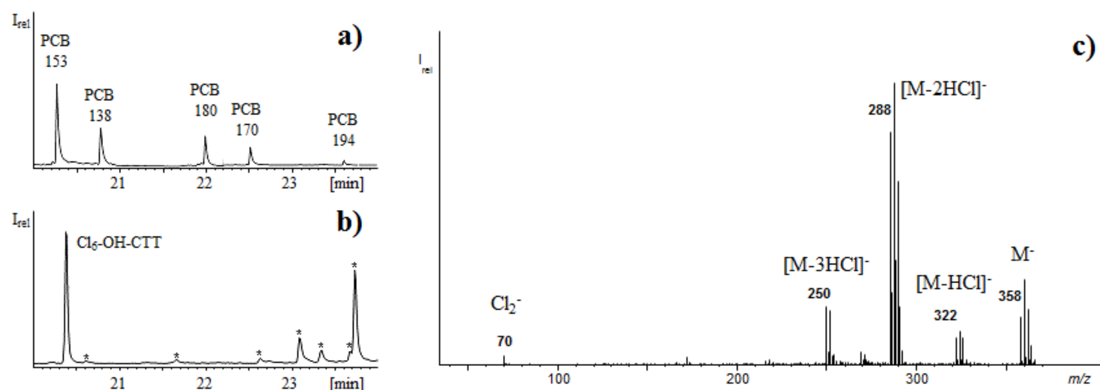


**Figure 3: Fractionation of PCBs and OH-CTTs on activated silica (column B) with (a) fraction B1 containing PCBs, (b) fraction B3 containing OH-CTTs (fraction B2 did neither contain PCBs nor OH-CTTs) and (c) GC/ECNI-MS full scan spectrum of the detected hexachlorinated OH-CTT.**

Different reasons may be responsible for the simple OH-CTT pattern (only one congener detected) and the rather low OH-CTT levels. First of all, polar bears are known to show a very simple toxaphene pattern with only very few recalcitrant congeners. On one hand this can be attributed to the efficient metabolism system of the polar bears [8]. On the other hand, polar bears predominantly feed on (ringed) seals [20]. Seals, in turn, are also able to metabolize most CTTs because the CTT residue is dominated by two major recalcitrant CTTs - B8-1413 and B9-1679 [10][11] - along with a few less abundant and partly metabolizable CTTs [22]. Hence it was not surprising that the number of OH-CTTs was low (although we had expected more than one OH-CTT). Last not least, the present polar bears were extremely nourished upon their arrival in Iceland which might have an impact on both the concentration level of POPs and their metabolites [20].
Liver samples of other marine mammals were not available to us. However, two cod livers analyzed in the same way did not show any OH-CTTs. This indicates that more powerful enzyme systems of marine mammals are required for the generation of OH-CTTs. The relevance of *in-vitro* incubation experiments performed some decades ago [13][14][15] which indicated the formation of OH-CTTs was fully confirmed in our study.

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