COMPOUND SPECIFIC ISOTOPE ANALYSIS INDICATES ABIOTIC CHLORINATION AS FORMATION MECHANISM OF *tris*(4-CHLOROPHENYL)METHANE

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Introduction

Tris-(4-chlorophenyl)methane (TCPMe) is a persistent halogenated compound ubiquitously present in the environment.[1] TCPMe has already been detected worldwide in biota, especially at higher trophic levels such as marine mammals at concentrations in the range <0.5-1500 ng/g lipid weight[1-4] or even in human tissues at concentrations in the range 1-30 ng/g lipid weight. Extreme high concentrations of up to 6800 ng/g lipid weight were detected in polar bear liver.[3]

Although the presence of TCPMe in different environmental compartments is well documented, there is a lack of knowledge on industrial production figures, origin and eventual application.[1] Buser suggested a link between environmental TCPMe and the insecticide DDT[5] by observing the formation of TCPMe during conditions similar to the technical synthesis of DDT and by detecting traces of TCPMe in technical DDT. Furthermore, correlation between concentration of *p,p'*-DDT and TCPMe is also often presented as indication of a possible common source.[4, 6] These findings point to the technical synthesis of DDT as a possible source of environmental TCPMe, but do not provide a clear explanation for the additional mono- to tri-chloro congeners observed in the environment.[7] Moreover, concentrations of *p,p'*-DDT and TCPMe do not correlate in all samples[4] and it is well known that concentrations of different lipophilic halogenated contaminants in lipid reservoirs significantly correlate without implying a common source (e.g., *p,p'*-DDE and PCB 153 in Greenland seal, R = 0.977[8]). Consequently, further investigations are necessary to definitively identify the source of TCPMe.

Compound-specific isotope analysis (CSIA) of chlorine can be applied to reveal if the chlorination reactions leading to a certain compound are abiotic or enzyme-catalyzed (i.e., biotic). In the first case a δ^{37}Cl of -4 to 0‰ [9] is observed for the formed compound, whereas a δ^{37}Cl in the range -13 to -10‰ should be expected for enzyme-catalyzed formation.[10] The aim of this work was to apply chlorine CSIA to investigate if TCPMe present in marine biota is formed by abiotic or by enzymatic chlorination. To the best of our knowledge this is the first attempt to identify the source of TCPMe by CSIA.

Methods and Materials

Sample. Blubber was collected from two Grey seals (*Halichoerus grypus*, approximately 3 and 6 years old) during autopsy of stranded specimens that had been sent to the Swedish Museum of Natural History. These seals were found deceased in 2003 on the Swedish east coast of the Bothnian Sea. The blubber was wrapped in aluminum foil, packed in polyethylene plastic bags and kept at -18 °C until extraction and analysis.

Sample clean-up. 5 kg of blubber were homogenized, filtered through a 1 mm mesh net, and centrifuged to obtain ca 3.2 l of clear liquid lipid phase. Aromatic compounds were extracted by continuous partitioning with acetonitrile in two 1.6 l lipid batches, using a Wallenberg perforator.[11] Each batch was extracted for the duration of 3 days, changing the acetonitrile every 24 h. After this the extracts were pooled and the solvent was evaporated. This extraction method allowed removing approximately 85% of the lipids. The remaining lipids

were treated with sulfuric acids and partitioned with hexane. This was repeated until no change in color was observed in the hexane phase after addition of sulfuric acid. The extracts were pooled, the solvent was evaporated to 0.5 ml and the sample was eluted with 50 ml hexane through a column (7 cm height, 1 cm i.d.) filled with silica containing 40% of sulfuric acid (w/w). This procedure was repeated until no color change on the silica surface was observed. Finally, the solvent was evaporated and the sample was transferred on a small column (5 mm i.d.) filled with 1 g of silica (activated at 470 °C for 4 hours), which had been conditioned with 4 ml hexane. The sample was eluted with 6 ml hexane (fraction 1), 4 ml hexane/dichloromethane (4+1, fraction 2), and 4 ml of hexane/dichloromethane (1+1, fraction 3). TCPMe, DDT and DDE were isolated from fraction 2 by preparative capillary gas chromatography (pcGC).

Preparative capillary gas chromatography. The pcGC system consisted of a 6890N gas chromatograph equipped with a flame ionization detector (FID) and a 7683 series injector, all from Agilent Technologies (Palo Alto, CA, USA), combined with cold injection system (CIS) and a preparative fraction collector (PFC) from Gerstel GmbH (Mülheim an der Ruhr, Germany). The vial containing the sample was kept at 6 °C during the entire pcGC procedure to limit the evaporation of solvent from the vial during the 3-5 days needed to process the sample by pcGC, thus preventing problems caused by variation of peak width and retention times due to increasing sample concentration. The CIS injector was operated in "solvent vent" mode, with vent flow and vent pressure adjusted to 60 ml/min and 5 psi, respectively. The solvent venting time was 0.1 min. The temperature of the inlet was set to 90 °C for 0.1 min and then increased to 270 °C at a rate of 12 °C/s, kept isothermal for 2.5 min and increased again to 300 °C with 5 °C/s. The splitless time was 2 min and the injection volume was 5 µl in all experiments.

A "megabore" fused silica capillary column (60 m length, 0.53 mm i.d.) coated with 0.5 µm of VF-5MS (cross linked 5% phenyl methylpolysiloxane, Factor Four, Varian, Walnut Creek, CA, USA) was used with helium as carrier gas at a constant flow of 6.8 ml/min (38 cm/s). The pcGC oven temperature program was as follows: 90 °C isothermal for 2 min, then 8 °C/min to 300 °C, and isothermal for 2 min. Approximately 1% of the total amount, which was injected into the CIS, was diverted to the FID after the capillary column. The temperature of the FID was kept constant during all injections at 300 °C. Air and hydrogen flows in the FID were 400 ml/min and 40 ml/min, respectively, with nitrogen as make-up gas at 45 ml/min. The PFC switch temperature, as well as the transfer line temperature, was kept constant at 315 °C through all injections and the traps were kept at 6 °C by the PFC cooling unit.

The sample was repeatedly injected in order to harvest sufficient amounts of target compounds (equivalent to >10 µg of chlorine) for CSIA. The trapping time window started ca. 1 s before and ended ca. 1 s after the elution of TCPMe, DDT, and DDE. The retention time was frequently controlled, and the trapping windows were corrected if necessary. The isolated samples were rinsed from the glass traps 5 times with 200 µl of toluene.

Chlorine isotope analysis. The method of chlorine isotope analysis, by means of sealed-tube combustion in conjunction with thermal ionization mass spectrometry (TIMS), has been described previously.[12] Briefly, the DDE and DDT samples were split in three aliquots, each containing 15-30 µg organic chlorine, and transferred to separate borosilicate tubes. The TCPMe sample was not split due to its limited size of ca. 13 µg chlorine. The solvent was evaporated by a nitrogen gas stream, and 50 mg CuO were added. After connection to a vacuum manifold, the tubes were torch sealed while partially immersed in liquid nitrogen to prevent volatilization of the samples. The tubes were heated to 630 °C for 2 hours, cleaned with ethanol and water, opened by cutting off a 5 mm section at the top, and extracted with 2.5 ml H_2O during 5 h in an ultrasonic bath. The dissolved chloride was isolated by precipitation as AgCl, which was redissolved in 1 M NH_3 (aq) for conversion by ion exchange to CsCl. After treatment with activated carbon, the CsCl was analyzed for chloride concentration in ion chromatography. CsCl stock solution (Alfa Aesar, $\delta^{37}Cl$ = -0.62‰ relative to standard mean ocean chloride (SMOC) was analyzed directly in TIMS for use as reference material in the calculation of $\delta^{37}Cl$, corresponding to a $^{37}Cl/^{35}Cl$ of 0.319410 for SMOC.

Each TIMS measurement was performed on 3 µg of chloride dissolved in 1 µl H_2O, mixed with 1.1-2.0 µl graphite slurry in 80% ethanol (50 mg graphite/ml slurry) on a tantalum filament. The varying graphite load was used to compensate for individual sample characteristics. A Finnigan MAT261 operated at 8.8 kV was used to obtain 336 ratios of masses 303/301 ($^{133}Cs_2{}^{37}Cl^+/^{133}Cs_2{}^{35}Cl^+$) with simultaneous twin Faraday-cup detection. The measured ratio 303/301 is directly proportional to the $^{37}Cl/^{35}Cl$ ratio of the sample. A check was performed on the ratio of masses 133/301 ($^{133}Cs^+/^{133}Cs_2{}^{35}Cl^+$) at the end of each measurement. Results with associated 133/301 ratios in the interval 1.0-1.8 were judged to be reliable for environmental-matrix samples.[12, 13]

Results and Discussion

Isolation of TCPMe by pcGC and identification by high resolurion mass spectrometry. Preparative capillary gas chromatography confirmed to be a well-suited technique for the isolation of semi-volatile trace compounds from complex mixtures, such as a seal blubber extract (Figure 1A). HRGC-EI-MS analysis showed a purity of >96% of the isolated TCPMe (Figure 1B). The elemental composition of the isolated TCPMe was confirmed by high resolution mass spectrometry. The measured mass of the most abundant isotope of the molecular ion was 346.0083 amu, deviating by only 0.5 ppm relatively to the theoretical mass of the molecular formula $C_{19}H_{13}Cl_3$. The electron ionization low resolution mass spectrum of the isolated compound in shown in Figure 1C.

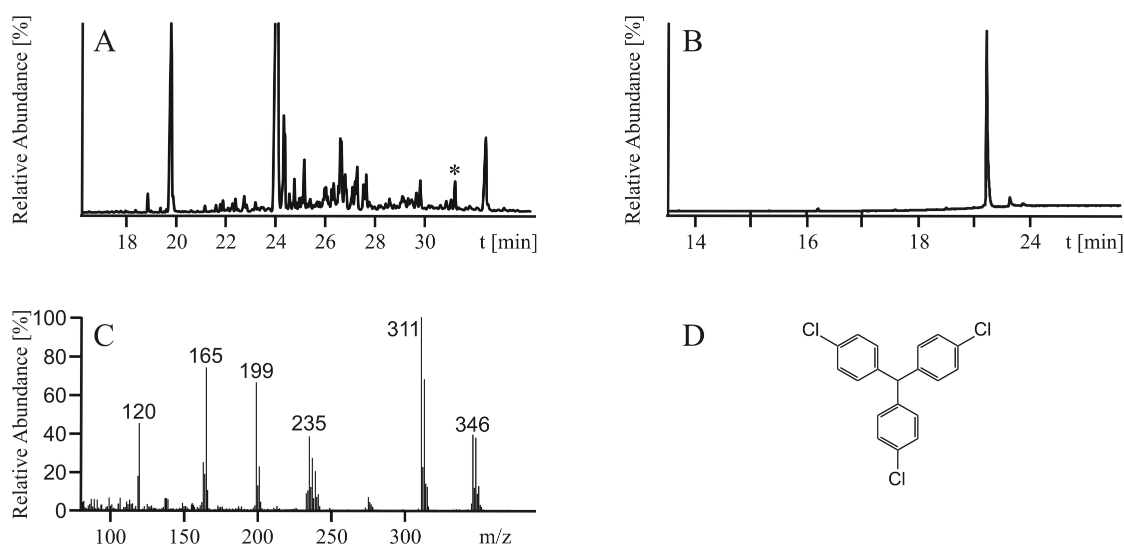

Figure 1. GC-FID chromatogram of the seal blubber extract containing TCPMe, which is marked with an asterisk (A) and HRGC-EI-MS chromatogram of the isolated TCPMe obtained by scanning the range 50-800 *m/z* (B). A and B were obtained with different HRGC setups. Electron ionization mass spectrum (C) and chemical structure (D) of the isolated TCPMe.

Chlorine isotope analysis. The results of the chlorine isotope analysis of TCPMe, DDT, and DDE isolated from the Baltic seal blubber are summarized in Table 1. The measured $\delta^{37}Cl$ value of TCPMe indicates abiotic chlorination as formation pathway. It was also highly consistent with the $\delta^{37}Cl$ previously reported for technical DDT and fairly consistent with the $\delta^{37}Cl$ of *p,p'*-DDT.[9] Although this result supports the hypothesis of a common origin of environmental TCPMe and DDT,[5] it still is not a definitive proof.

The $\delta^{37}Cl$ values observed for *p,p'*-DDT and *p,p'*-DDE in the same sample were higher, because of chlorine isotope fractionation during the degradation of *p,p'*-DDT to *p,p'*-DDE. This means that the C-^{35}Cl bond is broken at higher rate than the corresponding C-^{37}Cl bond, thus the $\delta^{37}Cl$ of the remaining *p,p'*-DDT is higher

than the δ^{37}Cl of the *p,p'*-DDT originally released into the environment.[13] The δ^{37}Cl of *p,p'*-DDE is also slightly higher than that of the *p,p'*-DDT released into the environment, because the elimination of $H^{35}Cl$ is kinetically favored over the elimination of $H^{37}Cl$, therefore the so formed *p,p'*-DDE is enriched in ^{37}Cl.[13] An analogous change in the δ^{37}Cl of TCPMe during its degradation in the environment cannot be expected because the main (known) degradation of TCPMe in the environment is hydroxylation to *tris*-(4-chlrophenyl)methanol (TCPMOH). This reaction does not involve chlorine, therefore no chlorine isotopic fractionation is observed and the TCPMe pool present in the environment still has the chlorine isotope ratio imprinted during its formation.

Table 1. δ^{37}Cl measured of TCPMe, *p,p*-DDT and *p,p'*-DDE obtained from the same seal blubber sample and compared to δ^{37}Cl of commercially available *p,p'*-DDT and technical DDT.

Compound	Origin	δ^{37}Cl [‰]	Lit.
TCPMe	Baltic seal blubber	-3.47 ± 0.5	-
p,p'-DDT	Baltic seal blubber	-0.69 ± 0.21	13
p,p'-DDE	Baltic seal blubber	-2.98 ± 0.57	13
p,p'-DDT	Aldrich	-4.34	10
Technical DDT	Ultra Scientific	-3.49	9

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References

1. de Boer, J. In *The Handbook of Environmental Chemistry*; Paasivirta, J. (ed.), Springer Verlag, Berlin Heidelberg, 2000; 3:31.
2. Watanabe M, Tanabe S, Miyazaki N, Petrov EA, Harman WM. *Mar Pollut Bul.* 1999;39:393.
3. Jarman WM, Simon M, Norstrom RJ, Burns SA, Bacon CA, Simonelt BRT, Risebrough RW. *Environ. Sci. Technol.* 1992;26:1770.
4. Falandysz J, Strandberg B, Strandberg L, Rappe C. *Environ Sci Technol* 1999;33:517.
5. Buser HR *Environ Sci Technol* 1995;29:2133.
6. Minh NH, Someya M, Minh TB, Kunisue T, Iwata H, Watanabe M, Tanabe S, Viet PH, Tuyen BC. *Environ Pollut* 2004;129:431.
7. Schwarzbauer J, Franke S, Franke W. *Fres J Anal Chem* 1999;365:529.
8. Oehme M, Schlabach M, Hummert K, Luckas B, Nordoy ES. *Sci Total Environ* 1995;162:75.
9. Drenzek NJ, Tarr CH, Eglinto TI, Heraty LJ, Sturchio NC, Shiner VJ, Reddy CM. *Org Geochem* 2002;33:437.
10. Reddy CM, Drenzek NJ, Eglinton TI, Heraty LJ, Sturchio NC, Shiner VJ. *J Am Chem Soc* 2002;124:14526.
11. Jensen S, Athanasiadou M, Berman Å. *Organohalogen Comp* 1992;8:79.
12. Holmstrand H, Andersson P, Gustafsson Ö. *Anal Chem* 2004;76:2336.
13. Holmstrand H, Mandalakis M, Zencak Z, Andersson P, Gustafsson Ö. *Environ Sci Technol* 2006, submitted.