ANALYSIS OF HEXABROMOCYCLODODECANES AND THEIR HYDROXY METABOLITES FROM *In vitro* AND ENVIRONMENTAL SAMPLES BY LC-MSMS

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Introduction

Brominated flame retardants (BFRs) are compounds added or applied to a wide range of commercial materials to improve flame resistance. Brominated flame retardants (BFRs) are high-production-volume chemicals with annual global demand of 204,000 tonnes (t) in 2001[1]. Hexabromocyclododecane (HBCD) is the third most widely used BFR after decabromo diphenyl ether (deca-BDE) and tetrabromobisphenol A (TBBP-A). The annual consumption of HBCD totalled 16,700 tonnes in 2001. HBCD ($C_{12}H_{18}Br_6$) is a brominated alicyclic hydrocarbon containing 74.7% bromine by weight. HBCD is synthesized by bromination of cis-trans-1,5,9-cyclododecatriene[2]. The resulting technical HBCD mixture contains three enantiomeric pairs; diastereomers termed α-, β-, and γ-HBCD which exist in rations of 6%, 8% and 80%, respectively. Theoretically 16 stereoisomers (6 enantiomeric pairs and 4 meso forms) may be formed at the production of HBCD[3]. HBCD is used primarily in polystyrene foams and thermal insulations in building materials but it is also used for upholstery textiles, draperies and wall coverings. HBCD is used as an additive BFR and therefore it may migrate from such products to the environment over the lifetime of the product. Concentrations of BFRs, including HBCD, have been increasing in many environmental compartments[4,5].

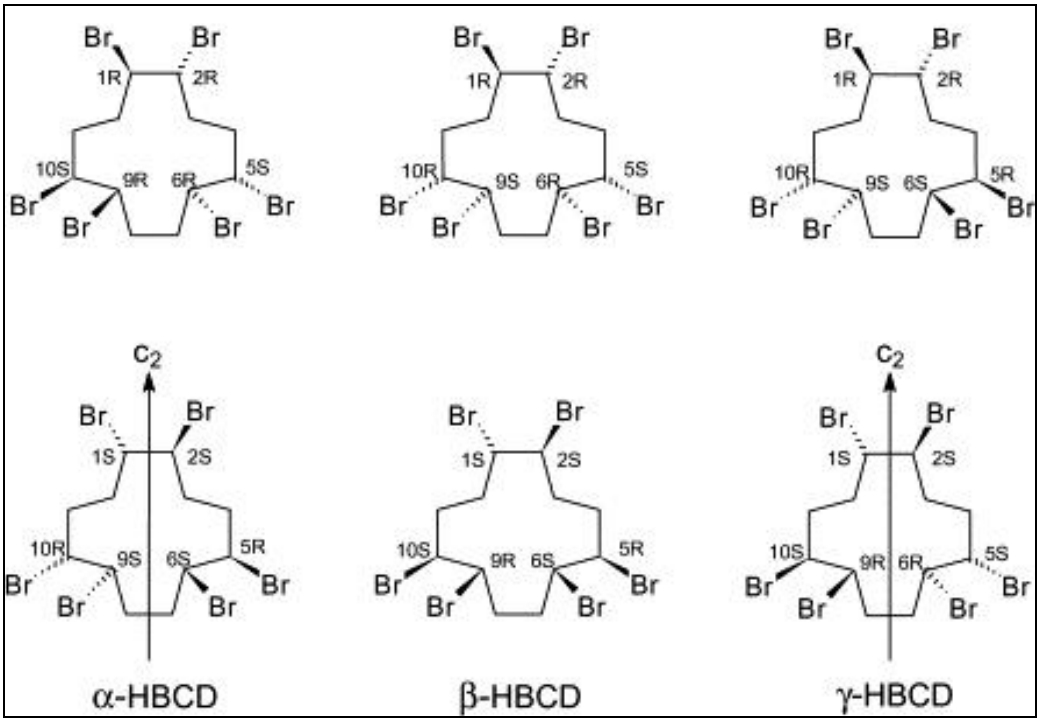

Figure. 1. Stereochemical structures of three enantiomeric pairs of diastereomers of 1,2,5,6,9,10-hexabromocyclododecane: α (left), β (middle) and γ (right)[6]

Materials and Methods

Hepatocyte assay.
In the hepatocyte assay freshly separated hepatocytes of juvenile rainbow trout (*Oncorhyncus mykiss*) were isolated by collagenase perfusion according to a slightly modified method of Moon et al[7]. Medium 199 (Sigma) with added L-glutamine, $NaHCO_3$, Na_2HPO_4 and an antibiotic-antimycotic solution (Sigma), was used for the washing and incubation medium. The viability of isolated hepatocytes was assessed by the Trypan Blue exclusion method, and only preparations exceeding 90% viability were accepted for the tests. The hepatocytes were diluted to a concentration of 1 x 10^6 cells ml^{-1} and distributed into polystyrene well plates in a final volume of 3 ml. For each test concentration the cells were incubated in triplicate for 72 hours at 12°C. Exposure concentrations were 6.3, 12.5, 25 and 50 µg/l.

Microsome assay.
Fish liver microsomes were used to metabolize the α-, β- and γ-HBCD *in vitro*. Chemicals were incubated in the presence of phosphate buffer pH 7.8, enzyme cofactor NADPH and fish liver microsomes at 20°C. Microsomes were prepared from rainbow trout (*Oncorhyncus mykiss*) after β-naphtoflavone treatment (50 mg/kg i.p.). Beta-naphtoflavone is a well-known enzyme inducer of mixed function oxygenase sub-family CYP1A1 in fish. CYP1A1 is of specific interest in fish for studies of the effect of xenobiotics. Samples were taken from the incubation mixture at 30 min time intervals for 120 min, and transferred to vials containing dichloromethane to stop the reaction and extract the parent compound and possible metabolites. In order to verify the enzymatic origin of the metabolites blank samples containing no enzyme or cofactor were run in parallel. In addition, samples without the test chemicals were analyzed as reagent blanks.

Analytical method.
Samples were extracted with dichloromethane (3 × 2.0 ml). Anhydrous sodium sulphate and Florisil (activated at 600°C, deactivated with 1.2% water) were used for sample clean up. Extracts were concentrated to nearly dryness under nitrogen and the solvent was changed to methanol.
Liquid chromatography - tandem mass spectrometry (LC-MS/MS) in the negative ion mode has been found to be the most suitable method for the isomer specific determination of HBCD[8,9]. Agilent 1100 series liquid chromatography system coupled to a Sciex API 4000 Qtrap hybrid triple quadrupole/linear ion trap mass spectrometer was used for all analyses.

Baseline separation of three diastereomers (α-, β-, and γ-HBCD) was achieved on the Vydac C18 polymeric column (2.1 mm × 150 mm) with methanol/water/acetonitrile mobile phase at a constant 250 µl/min flow rate. The chiral separation of the six HBCD enantiomers was achieved on the Macherey-Nagel NUCLEODEX β-PM column (4.0 mm × 200 mm) with methanol/water/acetonitrile mobile phase at a constant 500 µl/min flow rate[10].

The quantitative analyzes were done with electrospray ionization (ESI) while MS/MS detection employed multiple reaction monitoring (MRM) of the $[M-H]^-$ (m/z 640.6) to Br^- (m/z 78.9 and 80.9) transitions for native compounds and $[M+O]^-$ (m/z 656.6) to Br^- (m/z 78.9 and 80.9) transitions for the metabolites. Mass-labelled diastereomers (Wellington Laboratories) were used as internal standards. The qualitative determinations were done using the Linear Ion Trap (LIT) function of the instrument.

Results and Discussion

Sediment and fish samples collected from same locations were analysed. Our results confirm the earlier findings that there is isomeric selective partitioning of HBCD in different environmental compartments[10,11,12,13]. The distribution of the HBCD diastereomers in biota is clearly different that in technical mixtures or in sediments (Table 1, Figures 2 and 3).

	α -HBCD	β-HBCD	γ-HBCD
Lake Pyhäjärvi - sediment* pg/g (d.w.)	96	38	111
Lake Pyhäjärvi - pike* pg/g (l.w.)	7.1	0.21	0.15
Oulu - sediment** pg/g (d.w.)	0.73	0.43	0.72
Oulu - pike** pg/g (l.w.)	0.59	0.07	0.02
Kemi - sediment** pg/g (d.w.)	3.02	1.10	5.27
Kemi - pike** pg/g (l.w.)	1.6	0.26	0.17
River Vantaa – sediment pg/g (dw)	192	122	737
River Vantaa - pike pg/g (lw)	4.2	0.34	0.07
* Figures 2. and 3 ** Samples are collected from the Baltic Sea from the shore of the towns in question			

Table 1. Determined α-, β-, and γ-HBCD concentrations in sediments and fish (pike) samples.

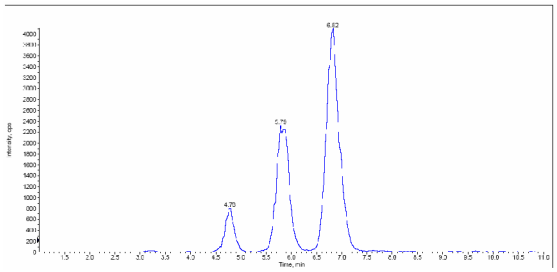

Figure 2. HBCD profile from sediment sample.

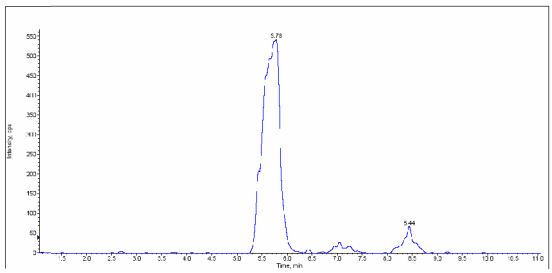

Figure 3. HBCD profile from pike sample.

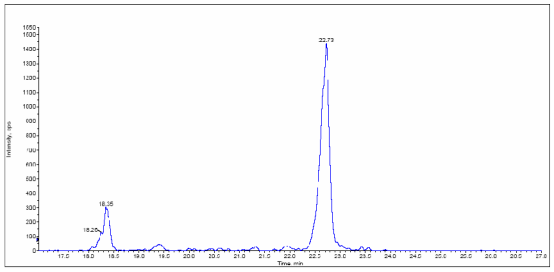

Figure 4. Chiral separation of OH- metabolites of γ-HBCD enantiomers from cell samples.
MRM transition: $[M+O]^-$ (656.6) to Br^- (78.9)

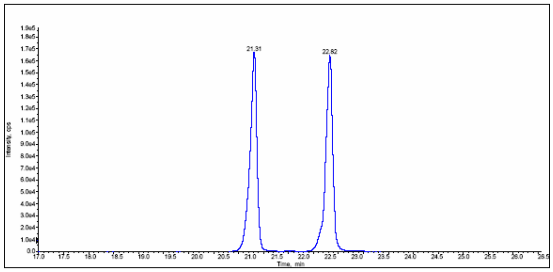

Figure 5. Chiral separation γ-HBCD enantiomers from cell samples.
MRM transition: $[M-H]^-$ (640.6) to Br^- (78.9)

Hydroxy metabolites were detected in the higher concentration and longer reaction time in the *in vitro* assay samples. The remarkable feature of the results is the clear differences at the rates of metabolism between three studied HBCD diastereomers (α-, β-, and γ-HBCD). The rates of metabolism of studied HBCD diastereomers in the increasing order is alpha < gamma < beta which may explain the enrichment of α-HBCD in aquatic species.

Conclusions

With the analytical methods used, the separation of the three HBCD diastereomers and the six HBCD enantiomers was achieved. The HBCD diastereomers (α-, β-, and γ-HBCD) are distributed differently in different environmental compartments *i.e.* fish versus sediment.
Hydroxy metabolites were detected from hepatocyte and enzyme preparate samples. The different rates of metabolism of three HBCD diastereomers could be seen from hepatocyte and microsome assays.
Also the metabolism rate of two chiral forms of the same diastereomer was found to be different.
By using hepatocytes or enzymatic (CYP1A1) reactions it will be possible to assess and predict environmentally relevant chemical isomers. In this respect it is important to use cells/preparates of environmentally relevant species.

References

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