

LC-MS/MS analysis of Hexabromocyclododecane (HBCD) isomers and Tetrabromobisphenol A (TBBPA) and levels in Danish fish for food consumption

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Introduction.

Brominated flame retardants are used in electronic equipment, plastics, textiles and building materials. HBCD is a technical mixture of isomers of 1,2,5,6,9,10 hexabromocyclododecane. While HBCD is mixed with the polymers of the products, tetrabromobisphenol A (TBBPA) is covalently bond and not released as easily [1]. As other flame retardants like formulations of penta- and octabrominated diphenylethers (PBDE) are phased out, the HBCD and TBBPA have received greater attention. EU is undertaking an ongoing evaluation of HBCD as the impacts on humans are not well known and HBCD mainly is used in Europe compared to the US and Asia [1]. The substances are present in the environment, e.g. in air, dust, wastewater, sewage sludge, plants and animals [2]. They are relatively persistent and bioaccumulates e.g. in fish and meat [3]. For that reason the Danish Food Institute wants to estimate the dietary intake of the flame retardants. An LC-MS/MS method was developed for the relatively polar flame retardants TBBPA and α -, β -, γ -HBCD.

Materials and Methods.

Standards. α -, β -, γ -Hexabromocyclododecane and Tetrabrombisphenol A as well as $^{13}\text{C}_{12}$ - α -HBCD and $^{13}\text{C}_{12}$ -TBBPA solutions 50 ± 2.5 $\mu\text{g/ml}$ were purchased from Wellington laboratories. Calibration standards of 0.25-50 ng/ml α -HBCD; 0.25-10 ng/ml β -, γ -HBCD and TBBPA and 10 ng/ml internal standards in methanol:water (4:1) were used for the calibrations bracketing the samples.

Sample preparation. 2-10 g homogenised fish sample was mixed with 60 g anhydrous sodium sulphate and soxhlet extracted for seven hours with 150 ml acetone:hexane 1:1. The extract was evaporated until a clear residue of lipid remains. In case any water was observed it could be removed by addition of ethanol:hexane 4:30 and evaporation of the azeotrop mixture. The lipid content was determined by weighing. App. 500 mg lipid was dissolved in 2.0 ml internal standard solution in hexane and cleaned up in 10 ml concentrated sulphuric acid by turning the centrifuge tube 20 times. After centrifuging the cleaned hexane phase was further cleaned with water and evaporated using a gentle stream of nitrogen. The sample was then dissolved in 200 μl methanol:water (4:1) and transferred to HPLC vials.

LC-MS/MS detection. The detection was performed on an Agilent HPLC coupled through an Electrospray interfaced to a Micromass Quatro Ultima tandem mass spectrometer. The separation of 20 μ l of the analytes was made on a Gemini 3 μ -C18 HPLC column (2 mm x 200 mm) using a gradient of methanol and 0.01% acetic acid at a flow of 0.2 ml/min. The source temperature was set to 120°C and the desolvation temperature to 380°C. The collision energy was 35 eV and the Argon collision gas pressure 2.3 x 10⁻³ mbar.

Results and discussion.

Figure 1 shows LC-MS/MS chromatograms of a salmon sample spiked with 1 ng/g ww of TBBPA and α -, β -, γ -HBCD including internal standards. Following MRM fragmentations in the negative ion mode were detected:

	Quantification	Verification
TBBPA	541.7>419.8	541.7>447.8
¹³ C ₁₂ -TBBPA	553.7>428.8	541.7>457.8
α -, β -, γ -HBCD	640.7>81	638.7>81
¹³ C ₁₂ - α -HBCD	653.6>81	651.6>81

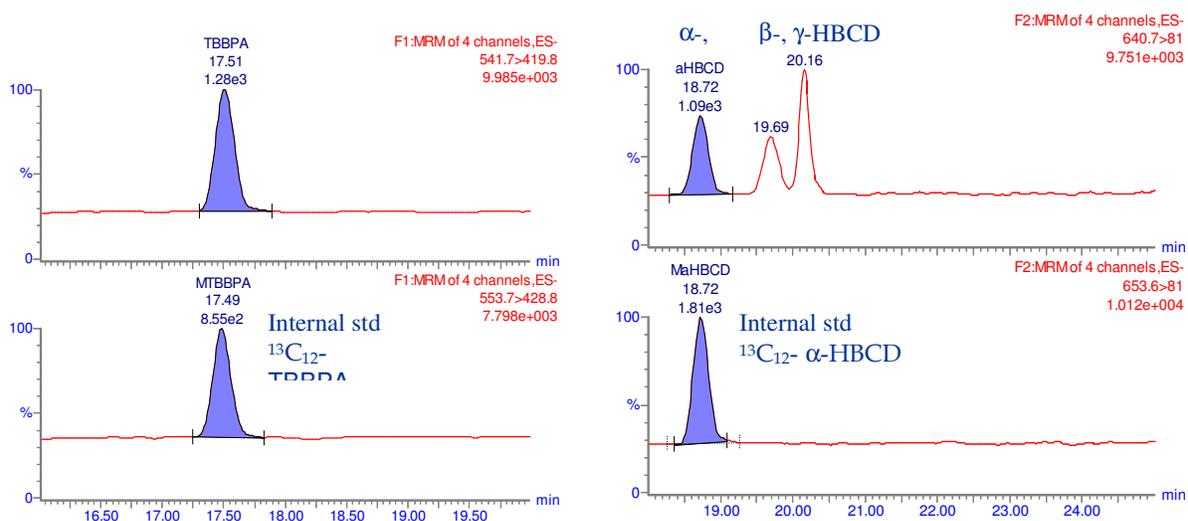


Figure 1. MRM traces of a salmon sample spiked with 1 ng/g ww of TBBPA and α -, β -, γ -HBCD.

Validation of the analytical method. The analytical method has been validated by performing recoveries of different fish samples spiked at four different spiking levels from 1-12 ng/g wet weight. The recoveries shown in Table 1 are slightly more accurate and precise at the higher spiking levels compared to the level of 1 ng/g ww. Furthermore the recoveries for α -HBCD

and TBBPA are better than for β -HBCD and γ -HBCD. This is explained by the use of labelled internal standards for the first mentioned analytes, whereas β -HBCD and γ -HBCD are measured relative to the α -HBCD labelled internal standard. However, as the α -HBCD most often contribute with >90% to the sum of the three isomers (Table 2), an improvement of the recoveries for β -HBCD and γ -HBCD is not supposed to significantly affect the results of Σ HBCD.

Table 1. Recoveries of HBCD isomers and TBBPA after spiking of different fish samples at spiking levels from 1-12 ng/g wet weight.

spiking level ng/g ww	α -HBCD mean \pm SD	β -HBCD mean \pm SD	γ -HBCD mean \pm SD	TBBPA mean \pm SD
1 ng/g, n=8	95 \pm 17	68 \pm 12	69 \pm 16	83 \pm 7
3-5 ng/g, n=9	95 \pm 9*	74 \pm 11	76 \pm 14	79 \pm 7
10-12 ng/g, n=5	108 \pm 8	87 \pm 11	92 \pm 10	93 \pm 6

* Recovery of α -HBCD of one sample is excluded as the actual background level turned out to be three times higher than the spiking level.

Double determinations of different types of real samples have been made in each series of app. eight fish samples, some within the same series and some repeated in another series. Within the data, the difference between series did not differ compared to the difference within a series and for that reason the data are pooled. The relative standard variation of double determinations was on average 15% for concentration levels < 0.5 ng/g ww (n=8) and 5% for concentration levels > 1 to 16 ng/g ww (n=12). The detection limits are often calculated as three times the standard deviations of blanks or samples spiked at low levels. However, since the background level of at least α -HBCD is relatively high, a rough estimate of the detection limit is instead made based on the level of the lowest calibration standard of 0.25 ng/ml (~ 0.1 ng/g lipid or 0.01 ng/g wet weight fish if the lipid content is 5-10 %).

Concentrations in Danish fish for consumption. The samples for this project were selected from stored fish homogenates which previously have been used for determinations of dioxins and PCB. The fish has been collected from Danish catching areas of the Baltic Sea and North Sea in 2002, 2003 and 2006 as well as from fish farms in 2006. Most of the samples were pools of 3 to 10 individual fish from the same age group except for the salmons from the Baltic Sea that were individual fish. The samples were stored at -20° until analysis for HBCD isomers and TBBPA.

The results show that α -HBCD was present in the highest amounts, followed by γ -HBCD and β -HBCD. TBBPA was almost not present in the samples (Table 2). The highest levels of Σ HBCD were found in cod liver that is used e.g. for production of vitamin supply (Vitamin D). The cod liver contained 37-66% lipid and 11 ng/g ww Σ HBCD. Salmon being a fatty fish with lipid content from

10-23% contained the second largest contaminant levels 2.45 ng/g ww. The mackerel taken from the North Sea contained 23-29% lipid and an average Σ HBCD content of 0.93 ng/g ww.

The concentrations found are comparable to other studies. In a Swedish study herring from the Baltic Sea and the North Sea contained 21-58 ng/g lipid and 34-180 ng/g lipid [2]. In the present study the content in herring ranged from 7-110 ng/g lipid with an average of 36 ng/g lipid (Table 2).

Table 2. Concentrations of HBCD isomers and TBBPA in fish for food consumption.

Fish	n	α -HBCD ng/g ww	β -HBCD ng/g ww	γ -HBCD ng/g ww	TBBPA ng/g ww	Σ HBCD ng/g ww	Σ HBCD ng/g lipid	Σ HBCD max. ng/g ww	Σ HBCD Std.dev. ng/g ww
Cod, North Sea	1	0.02	0.00	0.00	0.00	0.02	19.2	0.02	
Cod liver, 1 North Sea, 10 Baltic Sea	11	10.7	0.02	0.35	0.00	11.0	22.4	16.7	4.79
Eel, farmed	3	1.17	0.00	0.03	0.00	1.21	15.2	1.78	0.82
Herring, 7 North Sea, 13 Baltic Sea	20	1.14	0.03	0.10	0.02	1.27	36.4	3.73	0.56
Mackerel, North Sea	13	0.91	0.01	0.02	0.00	0.93	3.5	6.35	1.64
Plaice, North Sea	4	0.003*	0.000	0.001*	0.00	0.004*	3.0	0.005	0.00
Salmon, Baltic Sea	9	2.32	0.02	0.10	0.00	2.45	22.0	3.57	0.58
Salmon, farmed	1	1.43	0.01	0.05	0.01	1.49	20.4	1.49	
Trout, farmed	1	0.28	0.04	0.03	0.00	0.35	18.0	0.35	

* < LOD (0.01 ng/g ww)

Conclusions.

A sensitive LC-MS/MS method for determination of α -, β -, γ -HBCD and TBBPA has been developed. Based on recovery tests and real double determinations the method performs acceptable with respect to accuracy and precision. Preliminary results of 63 fish samples for food consumption show Σ HBCD contents from <0.01-16.7 ng/g ww or <0.1-110 ng/g lipid.

References.

1. The European Food Safety Authority <http://www.efsa.eu.int> The EFSA journal (2006) 328, 1-4.
2. Remberger M, Sternbeck J, Palm A, Kaj L, Strömberg K and Brorström-Lundén E 2004. The environmental occurrence of hexabromocyclododecane in Sweden, *Chemosphere* 54 9-21.
3. Morris S, Allchin CR, Zegers BN et al. 2004. Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea Estuaries and aquatic food webs. *Environ. Sci. Technol.*, 38, 5497-5504.