ANALYTICAL CAPABILITIES OF ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY COULPED TO HIGH RESOLUTION ORBITRAP MASS SPECTROMETRY IN ANALYSIS OF HEXABROMOCYCLODODECANE DIASTEREOISOMERS

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Introduction

Brominated flame retardants (BFRs) include a wide range of chemicals added to plastics to inhibit their ignition and reduce the chance of fire. One of the most widely used BFRs is hexabromocyclododecane (HBCD) consisted mainly of *α*-, *β*-, and *γ*-diastereoisomers. The properties of HBCDs are similar to those of persistent organic pollutants (POPs), and it was ubiquitously found in different objects such as water, soil, sediments, fish, birds, mammals, and people[1,2]. The most extensively used methods for analysis of HBCD are gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS)[3]. Analytical capabilities of the GC based methods are limited in terms of sensitivity and inability to provide diastereoselective analysis due to the thermolabile nature of this compound. LC eliminates the problem of thermal interconversion of HBCDs during the analysis, providing effective chromatographic resolution of diastereoisomers. Selective detection by MS positions the LC-MS combination as a method of choice for analysis of HBCDs. The most frequently used detection technique in diastereoisomer–specific analysis of HBCD is low resolution (LR) tandem MS (MS-MS) operated in selected reaction monitoring (SRM) mode. However, the use of LR-based LC-MS methods for analysis of HBCD diastereoisomers is well known[4], application of high resolution MS (HRMS) could provide additional facilities in analysis of BFRs. One of the most perspective HRMS techniques is Orbitrap-MS analyser which has reached the status of a mainstream mass spectrometry technique[5]. The analytical performance of Orbitrap-MS can support a wide range of applications including the analysis of trace-level components in complex mixtures and provide the powerful advantages of HRMS such as high mass resolution and mass accuracy up to 1 ppm. Combination of high resolution (up to 140,000 full width half maximum (FWHM)) with a fast scan speed provides new perspectives in analysis of ultra-trace levels of emerging contaminants, such as BFRs in environmental samples.

The aim of this study was to develop a new analytical method for diastereomer-specific determination of HBCD in fish, utilizing Orbitrap-MS for selective and sensitive detection. The method was validated and applied to analysis of twenty five Baltic wild salmon tissue samples.

Materials and methods

Chemicals and materials: reference standards were obtained either from Cambridge Isotope Laboratories, Inc. (Andover, USA) and from AccuStandard, Inc. (New Haven, USA), and high purity grade solvents/reagents such as n-hexane, dichloromethane (dcm), methanol, acetonitrile, florisil, sulphuric acid and sodium sulphate were sourced from Lab-Scan (Glivice,Poland), Sigma-Aldrich ChemieGmbH (Buchs, Switzerland) or Acros (New Jersey, USA).

Baltic wild salmon samples: twenty five Baltic wild salmon specimens (thirteen male and twelve female) of various age, length and weight were collected during the spawning period in October 2012 from the Daugava and Venta rivers. Summarized biological characteristics of the analysed salmons are given in Table 3.

Sample extraction and clean-up: briefly, homogenized fish fillet sample aliquot of 5.0 g was mixed with anhydrous sodium sulphate and $^{13}C_{12}$-labeled *α*-, *β*- and *γ*-HBCD diastereoisomers were added. Sample was extracted with mixture of dcm/*n*-hexane (1:1, v/v) and the organic extract was treated with 37N sulphuric acid followed by sulphuric acid impregnated silica gel. Additional clean-up step included the Florisil column: after the addition of the sample extract, the column was washed with n-hexane (eluate was discarded) and the analytes of interest were eluted with dcm/n-hexane (1:1, v/v) mixture. The fraction was concentrated and reconstituted in 200 µL of mobile phase.

Instrumental analysis: UPLC-Orbitrap-HRMS system consisting of Thermo Accela UPLC system (Zwingen, Switzerland) coupled to an Orbitrap Q Exactive mass spectrometer (Bremen, Germany) equipped with heated electrospray ionization (HESI-II) interface was used operated in negative ion mode. During the tuning procedure infusion of the methanolic solution containing target compounds was performed and the signal was optimized for the highest response of the $[M–H]^-$ ion. Detection in targeted selected ion monitoring (t-SIM) mode was used for determination of selected compounds using the two $[M–H]^-$ most abundant ions of the respective molecular ion cluster for both the native and the $^{13}C_{12}$-labeled surrogates. The channels monitored for HBCD diastereoisomers were *m/z* 640.6374 (quantitation) and *m/z* 638.6396 (confirmation) for the native components, and *m/z* 652.6782 (quantitation) and *m/z* 650.6804 (confirmation) for the $^{13}C_{12}$-labeled surrogates. Detailed instrumental conditions are summarized in Table 1. UPLC separation of target compounds was carried out using Kinetex C18, 100 mm × 2.1 mm, 1.7 µm analytical column, applying a flow rate of 250 µL min^{-1} with mobile phase gradient based on (A) methanol-water (75:25, v/v) and (B) acetonitrile. The gradient began at the initial composition (A/B) of 20:80 (v/v) that was maintained for 1.0 min and then ramped to 55:45 over 0.1 min, where it was held for 6.0 min before returning to the initial conditions over 1.0 min. The injection volume of 10 µL was applied both for standard solutions and sample extracts.

Table 1
Orbitrap-MS instrumental conditions for determination of HBCD diastereoisomers.

HESI-II conditions	
Sheath gas flow	15 a.u.*
Auxiliary gas flow	5 a.u.*
Capillary temperature	250 °C
Source heater temperature	250 °C
Spray voltage	4.5 kV
S-lens radio frequency	50
Orbitrap-MS conditions	
Maximum injection time	100 ms
Automatic gain control (AGC target)	5×10^4
MS resolution	35 000 FWHM

a.u.* – arbitrary units

Quality assurance/quality control: analyte identification criteria were based on the retention times of native components and $^{13}C_{12}$-labeled surrogates (internal standards), and isotopic peak ratios of the monitored ions. The isotope ratio of two monitored ions (target/confirmation) was within 15% of the value obtained for the medium calibration point. Calibration curves over the concentration range 1.00–500 pg μL^{-1} were used for quantitation of HBCDs. Quantitation was carried out on the basis of stable isotope dilution with the $^{13}C_{12}$-labeled surrogates and internal standardization. Procedural blanks and quality control samples were analyzed in each sample sequence.

Results and discussion

Method performance: generally, extraction and clean-up steps were based on procedures reported earlier for analysis of HBCD in biota samples[3,4]. Additionaly, potential isobaric mass interferants (such as PBDEs) were removed with clean-up on a Florisil column. Moreover, three-stage sample clean-up procedure provided the final sample extracts of the desired purity, which was essential for the analysis of organobromine compounds, since the matrix components tended to adhere on the hot surfaces of the ion source and the thermally labile compounds such as HBCDs were destroyed on these spots resulting in a drop of method sensitivity. The absolute recoveries of the isotopicaly labeled internal standards were in the range of 70–110%. Three HBCD diastereomers were found to be chromatographically separated using methanol, acetonitrile, and water LC gradient with the absolute retention times of 3.89, 4.16, and 4.68 min for the α-, β- and γ-HBCD, respectively.

The Q Exactive mass analyzer consists of four main modules: quadrupole mass filter for precursor ion selection (enables targetted single ion monitorng mode (t-SIM)), intermediate storage device (C-Trap) for short pulse injection, collision cell for performing of HCD (Higher Energy Collisional Dissociation) experiments and Orbitrap analyzer for the Fourier transform mass analysis. After the ionization of analytes of interest the ions are transferred into the C-Trap through the quadrupole rods assembly which operates as ion transmission device. In the C-Trap, the ions are accumulated and then injected into the Orbitrap analyzer where mass spectra are acquired. In the case of operation in targeted-MS2 (t-MS2) mode, before the injection into the Orbitrap analyzer ions are passed through the C-Trap into the HCD cell where HCD takes place. In combination with the mass filter this allows MS/MS experiments or all ion fragmentation (AIF) in case of a broad range of selected *m/z* ratios. After all ions are fragmented in the HCD cell, they are transferred back into the C-Trap from where they are injected into the Orbitrap analyzer for detection.

Two different detection modes were attempted during the method development: t-MS2 and t-SIM modes. The t-MS2 mode was checked in order to achieve a potentially better selectivity of the method in comparison to t-SIM detection. This type of detection utilized the tandem MS and the fragmentation of the precursor ions of *m/z* 638.6396 and *m/z* 640.6374 in the HCD collision cell resulted in the intensive formation of *m/z* 78.9173 and *m/z* 80.9152 pair corresponding to $[Br]^-$ ions. The t-SIM mode did not rely on the tandem MS option and comprised a full MS scan with an adjusted mass resolution within a defined isolation window, and further registration of target ions with selected *m/z* values. Study results showed that the t-SIM method provided at least ten times lower LOQs in comparison to the t-MS2 method. The chromatograms of spiked butter fish sample (0.40 pg g^{-1} fresh weight (f.w.) of each diastereoisomer) analyzed in the t-MS2 and t-SIM modes are shown in Fig. 1. The decreased sensitivity of the t-MS2 mode can be explained by taking into account the losses/incomplete fragmentation of precursor ions during the collision process. During the analysis of spiked samples no selectivity problems were observed under the t-SIM conditions and this mode was considered to be more suitable with the registration of two specific fragments ($[M–H]^-$) both for the native and $^{13}C_{12}$-labeled HBCDs.

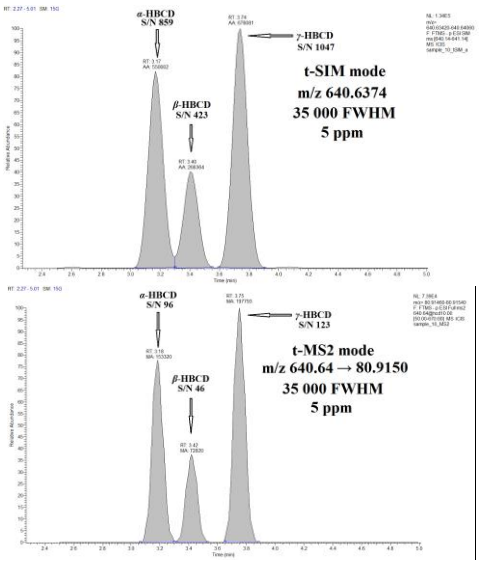

Fig. 1. The chromatograms of spiked butter fish sample analyzed in the t-MS2 and t-SIM modes.

Because of the reduced scanning speed provided by the Q Exactive-MS system at maximum possible mass resolution mode (140 000 FWHM), processing of the data with appropriate scan filter and smoothing of the raw chromatograms affects the sensitivity and reproducibility of the results compared to lower mass resolution modes. On the contrary, the application of lower resolution modes creates higher background noise and could lead to reduced signal-to-noise (S/N) ratios and false positive/negative results because of the degraded mass accuracy. On the basis of the experimental results a resolution of 35,000 FWHM was found to be optimal as a compromise between selectivity, sensitivity and reproducibility.

Validation: the performance of the method was evaluated by run-to-run (n = 5) and day-to-day (n = 3) analyses of spiked butter fish homogenate at three concentration levels which were selected taking into account the strong predominance of *α*-HBCD in comparison with *β*- and *γ*-HBCDs in real fish samples. Accuracy (recovery), repeatability (intra-day precision), intermediate precision (inter-day precision), instrumental and method limit of quantification (i-LOQ, and LOQ) were examined. The S/N ratio used for calculation of i-LOQ values was 10:1. The method LOQ was assessed by calculations taking into account the sample preparation procedure. The selectivity of the method was examined by analysing a procedural blank samples. The linearity values were calculated using six-point calibration curves. Detailed results of quality parameters of the method are summarized in Table 2. Validation results reveal that the elaborated methodology provided satisfactory reproducibility, linearity, and sensitivity for the analysis of HBCD diastereomers in fish samples.

Application: method was applied to the determination of HBCD diastereomers in tissue of twenty five salmon samples. The concentrations of individual HBCD diastereoisomers and the total HBCD for all analyzed samples are summarized in Table 3. The total HBCD concentrations within the samples ranged from 0.39 to 3.82 ng g^{-1}w.w.,with an average of 1.59 ng g^{-1}w.w. and were of similar magnitude to those detected in fish from Baltic Sea in other studies. Earlier studies report on the total-HBCD concentrations in Baltic salmon up to 4.85 ng g^{-1}w.w.[6] The diastereomer pattern typical for aquatic biota was observed with strongly pronounced domination of *α*-HBCD over *β*- and *γ*-HBCDs.

Acknowledgements
This work has been supported by the European Social Fund within the project "Support for Doctoral Studies at University of Latvia".

Table 2
Validation results for HBCD diastereomers.

Compound	Linearity of measurement, pg µL^{-1}	i-LOQ, pg on column	LOQ, ng g^{-1} f.w.	1st spiking level				2nd spiking level				3rd spiking level			
				Spiking level, ng g^{-1} f.w.	Recovery (n=3) [a], %	Intra-day precision, (n=3) [b], %	Inter-day precision (n=3) [c], %	Spiking level, ng g^{-1} f.w.	Recovery (n=3) [a], %	Intra-day precision, (n=3) [b], %	Inter-day precision (n=3) [c], %	Spiking level, ng g^{-1} f.w.	Recovery (n=3) [a], %	Intra-day precision, (n=3) [b], %	Inter-day precision (n=3) [c], %
***α*-HBCD**	1.00 – 500	1.5	0.006	0.50	103.6	3.9	4.4	1.00	102.5	7.2	12.5	1.50	102.5	4.6	5.2
***β*-HBCD**	1.00 – 500	3.0	0.012	0.06	102.7	7.1	7.7	0.12	102.4	2.9	3.0	0.18	101.1	1.6	1.6
***γ*-HBCD**	1.00 – 500	1.3	0.005	0.04	95.0	8.3	9.0	0.08	95.5	6.0	6.3	0.12	91.4	7.6	7.2

[a] – average recovery (%) for selected diastereomer at the corresponding fortification level calculated from the data obtained on three different days;
[b] – average intra-day precision (%) for selected diastereomer at the corresponding fortification level calculated from the data obtained on three different days;
[c] – average inter-day precision (%) for selected diastereomer at the corresponding fortification level calculated from the data obtained on three different days.

Table 3
Overview on the biological parameters and concentrations of HBCD diastereoisomers in Baltic wild salmon samples.

	Minimum	Maximum	Averegage
Length, cm	56	93	74
Weight, kg	2.0	11.3	5.3
Age, years	1	3	2
Concentration, ng g^{-1}f.w.			
***α*-HBCD**	0.36	3.67	1.46
***β*-HBCD**	<0.01	0.18	0.07
***γ*-HBCD**	0.02	0.15	0.07
Total-HBCD	0.39	3.82	1.59

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