Simultaneous Determination of α-, β- and γ-hexabromocyclododecane Diastereoisomers in Sewage Sludge using Liquid Chromatography Tandem Mass Spectrometry

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Hexabromocyclododecane (HBCD) is a large-scale usage brominated flame retardant consisting of a mixture of diastereoisomers which has been reported as an ubiquitous environmental contaminant. HBCD has attracted much attention due to its toxicity and increasing levels in waste water sewage sludge. Since November 2014, HBCD was added to the Persistent Organic Pollutants list of the Stockholm Convention which generated a higher concern regarding its presence in the environment. A rapid (8 min) and accurate liquid chromatography electrospray ionization tandem mass spectrometry method (LC-ESI-MS/MS) was developed to separate and detect α-, β- and γ-HBCD isomers from sewage sludge. Optimization of LC-MS parameters (column temperature, mobile phase composition and flow, collision energy, fragmentor voltage, capillary voltage and drying gas temperature) allowed complete separation of the three isomers and also very good detection sensitivity with instrumental quantitation limits (IQLs) between 0.3 and 0.6 ng/mL. The three isomers were separated on a C18 reversed-phase column, kept at 18°C, using a H_2O/ACN/MeOH mobile phase mixture and detected by negative electrospray ionization using MRM mode with two transitions, one for quantitation and one for confirmation. MS detector response was linear in the range 1.0 ÷ 100.0 ng/mL with correlation coefficients (R^2) higher than 0.99 for all three isomers. A simple ultrasonic assisted liquid-solid extraction procedure using a solvent mixture was employed to extract HBCD isomers from WWTP sewage sludge. Overall method intra-day and inter-day precision (RSD%) were situated between 7.6 ÷ 9.2% and 10.3 ÷ 14.5%, respectively. LOQs for α-, β- and γ-HBCD were 1.4, 0.7 and 1.0 ng/g, comparable and even lower to those reported by similar studies concerning HBCD presence in WWTP sewage sludge. HBCD presence was tested from several WWTP sludge samples. All three isomers were found with 100% detection frequency ranging from 3 to 76 ng/g.

Keywords: hexabromocyclododecane (HBCD), brominated flame retardants (BFRs), WWTP sewage sludge, ultrasonic assisted liquid-solid extraction, LC-MS/MS

Emerging organic contaminants represent newly discovered chemicals in all environmental factors. These compounds were not previously detected either due to lack of sensitive analytical instrumentation or because they were simply not targeted as pollutants at the respective time.

Emerging contaminants include, but are not limited to, pharmaceuticals and personal care products, pesticides, steroids and hormones, surfactants, food additives, industrial additives, flame retardants [1-5]. These compounds pose a serious threat to environment, human and wildlife health. Brominated flame retardants (BFRs), including hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDE), are widely used in a variety of commercial products to reduce the risk of fire by decreasing their inflammability [6]. One of the most used flame retardants is HBCD with almost 17,000 tons produced in 2001 worldwide. About 80% of the produced HBCD is used in the production of expanded (EPS) and extruded (XPS) polystyrene for the construction industry [7]. Recent studies indicate that HBCD isomers are ubiquitous organic contaminants and show similar characteristics to those of persistent organic pollutants (POPs): persistence, bioaccumulation and toxicity, which is why, since November 2014, HBCD was added to the Persistent Organic Pollutants Stockholm Convention list. As HBCD is not covalently bonded to the material that is added as an additive, it can easily be released into the environment [8]. Commercial HBCD mixtures consist primarily of three diastereomers, termed α-HBCD, β-HBCD, and γ-HBCD, the latter being the major constituent (approx. 70%) [9]. Due to their high hydrophobicity (log Kow > 5) and extremely low water solubility, hexabromocyclododecane is easily accumulated in fat tissue of biota and readily adsorbs to sediment particles, soil and sewage sludge. In 2013, EU legislation has established an environmental standard for surface water, where annual maximum amount of HBCD allowed should be 1.6 ng/L (sum of α, β and γ-HBCD) [10]. Maximum admissible concentrations of these compounds in soil, sediments or sludge are not currently established [11]. Analytical methods used for quantitation of these analytes in trace amounts from various environmental matrices are mainly LC-MS and GC-MS. GC-MS operated in negative chemical ionization mode (NCI) was used in the beginning as a sensitive method to determine HBCD, but is limited to the determination of the sum of HBCD isomers, because at temperatures higher than 160°C, the phenomena of isomer inter-conversion and thermo-degradation readily occur. Because of this, the best choice to determine HBCD isomers is LC-MS using either ESI or APCI ionization, since it allows both chromatographic

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separation and sensitive detection of α-HBCD, β-HBCD and γ-HBCD [12-15]. The aim of the present study was to develop, optimize and validate a sensitive, selective, and accurate LC-MS/MS method coupled to ultrasonic assisted liquid-solid extraction sample concentration technique able to extract, separate and detect α -HBCD, β-HBCD and γ-HBCD from WWTP sewage sludge at trace level concentration (ng/g) [16]. The method was applied to establish the occurrence of these HBCD isomers in sewage sludge coming from different waste water treatment plants located in Romania.

Experimental part

Reagents and chemicals

Individual high purity standards of α-HBCD, β-HBCD, γ-HBCD and isotopically labeled β-HBCD ($^{13}C_{12}$-β-HBCD) which was used as internal standard were purchased from Sigma-Aldrich. Methanol and acetonitrile of HPLC-grade purity were acquired from Merck. Dichloromethane, n-hexane, and acetone were purchased from Sigma-Aldrich.

LC-MS instrumentation and conditions. Experiments were performed using an Agilent 1260 series LC system (Waldbronn, Germany) consisting of: binary pump, autosampler, and thermostatted column compartment coupled with an Agilent 6410B triple-quadrupole mass spectrometer with electrospray ionization source (ESI). All chromatographic runs were carried out on a Hypersil Gold column (100 x 2.1 mm, 3.0µm) from Thermo Scientific which was kept at 18°C. All experiments were performed in isocratic elution conditions at a flow-rate of 0.25 mL/min. Mobile phase consisted of a ternary mixture of H_2O/ACN/MeOH = 20/40/40 (v/v) (v/v). Method injection volume was 5 ìL using MeOH as sample diluent. MS detection was achieved using Multiple Reaction Monitoring (MRM) acquisition mode. Full-Scan MS spectra was acquired in the range 70 - 700 Da to establish MRM transitions. Retention time, MRM transitions, fragmentor voltages, collision energies and other MS parameters are given in Table1. ESI ionization source was operated in negative mode with 300°C as the drying gas temperature, 10 L/min drying gas flow, 50 psi nebulizer pressure and 5000 V capillary voltage.

Results and discussions

LC separation optimization. The LC method was optimized in order to obtain an efficient separation of the three diastereoisomers with narrow peaks and high resolution between them in the shortest possible time. HBCD isomers are compounds with very high hydrophobicity (log Kow = 5.38, 5.47, and 5.80 respectively). Thus, the chosen mobile phase composition for the separation of the isomers was rich in organic solvent (80%). A short retention study was done using both ACN (60 ÷ 80%) and MeOH (70 ÷ 90%) as organic modifier and H_2O up to 100%. Afterwards, different mixtures between the two organic solvents (ACN : MeOH = 2 : 1, 1 : 1 and 1 : 2) were also tested to obtain the best separation. Mobile phase composition which proved to separate best the three isomers in the shortest period of time (8 min) was the ternary mixture H_2O/ACN/MeOH = 20/40/40 (v/v). It is worthwhile to mention that baseline resolution is a

Table 1

MS PARAMETERS FOR THE DETECTION OF HBCD ISOMERS AND LABELED INTERNAL STANDARD (MRM TRANSITIONS, COLLISION ENERGY, ETC.)

Compound	Molecular formula	Molecular mass	Retention time (min)	MRM transitions	Fragmentor voltage (V)	Collision energy (V)	Dwell time (msec)
α-HBCD	C12H18Br6	641.7	4.56	640.7 → 80.9 640.7 → 79.0	70	20	250
β-HBCD	C12H18Br6	641.7	5.36	640.7 → 80.9 640.7 → 79.0			
$^{13}C_{12}$-β-HBCD	*C12H18Br6	653.6	5.36	652.7 → 80.9 652.7 → 79.0			
γ-HBCD	C12H18Br6	641.7	7.04	640.7 → 80.9 640.7 → 79.0			

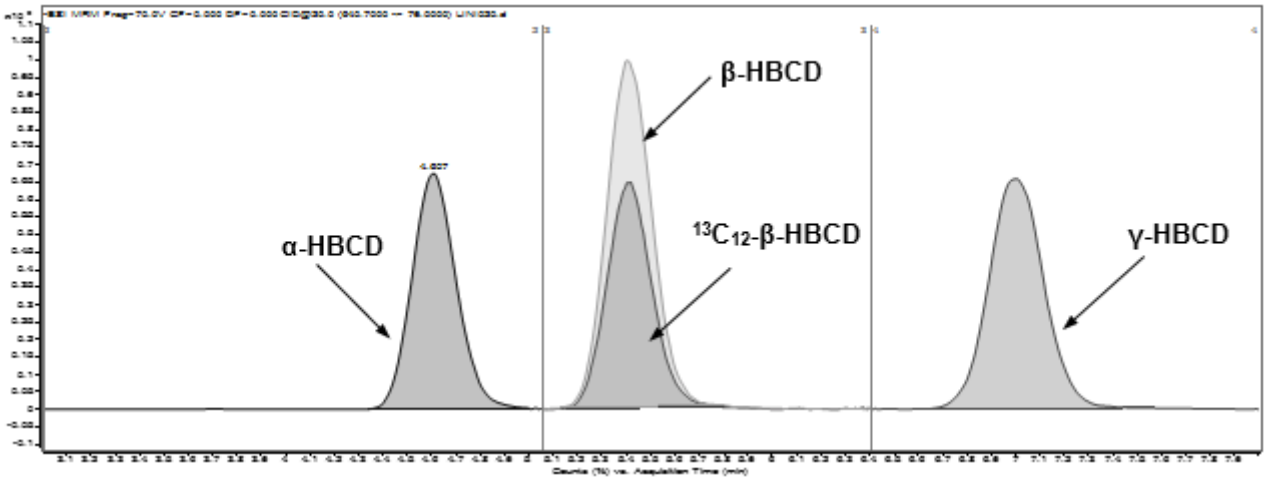

Fig 1. MRM overlaid extracted ion chromatograms of a mixed 50 µg/L methanol solution of the three HBCDs and the labeled $^{13}C_{12}$-β-HBCD internal standard

must for the three isomers to be detected individually due to the fact that they are isobaric molecules and present the same MRM transitions. Concerning the elution order of the HBCD isomers (table 1), it can be observed that these compounds elute in order of increasing hydrophobicity (log Kow increase) as it would be expected for solutes which interact with stationary phase solely by hydrophobic interactions (van der Waals interaction and hydrogen bonding) [17]. Indeed, the structure of the HBCD isomers allows only these two types of interaction with the stationary phase of the chromatographic column. Mobile phase flow-rate was modified between 0.2 and 0.4 mL/min with a 0.05 increment. The final chosen value was 0.25 mL/min because it allowed both fast separation and good MS sensitivity of the analytes, keeping in mind that ESI ionization is more efficient at lower flow-rates [18]. Column temperature was carefully chosen after a short thermodynamic study of analytes retention. Thus, column temperature was modified from 15 to 35°C to obtain better separation in terms of resolution between the first two eluting analytes, namely α-HBCD and β-HBCD isomers (fig. 1). All analytes and internal standard behaved normally upon temperature increase by a retention decrease (normal van't Hoff behaviour). The highest resolution was obtained at 18°C and allowed setting of 2 detection windows: one for α-HBCD and the other for β-HBCD and $^{13}C_{12}$-β-HBCD. The developed LC method resulted in at least baseline separation of the three isomers. Overall the optimized LC parameters allowed baseline separation of the 3 isomers in less than 8 min. $^{13}C_{12}$-β-HBCD co-eluted with β-HBCD but these analytes were selectively detected by different MS/MS transitions due to different molecular weight.

MS detection optimization

MS detection parameters were optimized to obtain maximum sensitivity for the HBCD isomers quantitation. Analytes ionization in the MS source was tested in both polarity modes (±). Due to electronegative Bromine atoms in their molecule, negative ionization is favored for these compounds by proton elimination. Thus, negative ESI ionization mode was further used. All ESI ionization source parameters were optimized by modification in a wide range to obtain highest ionization efficiency for the three HBCD isomers. Capillary voltage (4000 ÷ 6000V), drying gas temperature (275 ÷ 325°C), nebulizer pressure (40 ÷ 60 psi) and drying gas flow-rate (6 ÷ 10 L/min) were modified one at a time and the analyte response (peak area, S/N) was recorded. The final chosen values, giving maximum peak area response, were: 5000V capillary voltage, 300°C drying gas temperature, 50 psi nebulizer pressure and 10 L/min drying gas flow. These values were further used in method development. Collision energy (CE) applied in the collision cell (Q2) to the precursor ions to dissociate them and obtain product ions was varied also between 5 and 30 V with a 5 V increment. CE of 20 V generated highest fragmentation yield of the precursor ions for the target analytes and was chosen for the final method. Using the optimal collision energy values, the fragmentor voltage was also optimized in the range 50 - 150 V. A value of 70 V for the fragmentor voltage was found to generate highest transmission of ions to the quadrupoles and thus maximum sensitivity for all three analytes and the internal standard. After optimization of all mass spectrometric parameters, the instrumental quantitation limits (IQL) were determined to be between 0.31 and 0.62 ng/mL (table 2).

Table 2
INSTRUMENTAL QUANTITATION LIMITS DETERMINED FOR THE THREE ANALYTES

Compound	IQL (ng/mL)
α-HBCD	0.62
β-HBCD	0.31
γ-HBCD	0.40

Sample extraction. For the optimization of extraction and clean-up procedure, real WWTP sewage sludge samples were used. In the first step, the samples were freeze dried and then the lyophilized material was ground, homogenized, and stored in sealed containers at -20 °C until analysis. 0.5 g of dried sample was spiked target analytes mixture solution and also $^{13}C_{12}$-β-HBCD solution at up to a 50 ng/g. Extraction was carried out by sonication using 20 mL of dichloromethane : hexane (1:1, v/v), in two

Table 3
CORRELATION COEFFICIENTS (R^2), INTRA-DAY AND INTER-DAY PRECISION, ANALYTE RECOVERY, INSTRUMENTAL QUANTITATION LIMIT (IQL) AND METHOD QUANTITATION LIMIT (LOQ)

Analyte	R^2	Precision (%)		Overall method recovery (% ± RSD%)	IQL (µg/L)	LOQ (ng/g dw)
		Intra-day	Inter-day			
α-HBCD	0.9997	9.2	14.5	86 ± 9.2	0.62	1.4
β-HBCD	0.9996	7.6	10.3	85 ± 7.6	0.31	0.7
γ-HBCD	0.9989	8.3	13.0	79 ± 8.3	0.40	1.0

Table 4
CONCENTRATION OF HBCD ISOMERS IN 6 WWTP SEWAGE SLUDGE SAMPLES FROM ROMANIA

Sample	Concentration (ng/g, dw)		
	α-HBCD	β-HBCD	γ-HBCD
S1	42.7	10.5	38.0
S2	63.5	14.7	75.8
S3	7.8	3.0	7.1
S4	44.0	6.2	39.7
S5	37.8	6.0	39.7
S6	38.5	5.6	54.5

portions. Samples were sonicated for 15 min and the collected organic layers were dried on sodium sulphate and then filtered. The crude extracts were purified with a silica gel column and eluted with 30 mL DCM. Under a gentle nitrogen stream, organic phase was evaporated to dryness and re-dissolved with 2.0 mL of MeOH prior to LC-MS analysis.

LC-MS/MS method validation. To account for its performance, the developed LC-MS/MS method was validated with respect to specificity, linearity, precision, accuracy and limit of quantitation. MS detector response proved to be linear in the range 1 ÷ 100μg/L with high correlation coefficients ($R^2 > 0.998$). Instrumental limits of quantitation were determined by injecting decreasing concentrations of HBCD isomers solutions until a S/N of 10 was obtained (table 2). Intra-day and inter-day method precision was tested on 6 replicates by spiking 50 ng/g HBCD isomers mixture in lyophilized sewage sludge. RSD% values were situated between 7.6 – 9.2% for intra-day precision and 10.3 – 14.5% for inter-day precision, respectively. Method accuracy was tested also at 50 ng/g and the obtained analyte recovery was situated between 79 and 86% with internal standard correction as can be observed in table 3. Overall method LOQs were situated below 1.4 ng/g dry weight for each of the 3 isomers.

Hexabromocyclododecane isomers occurrence in WWTP sewage sludge. The developed method was used to determine the presence of the target compounds in sludge samples collected from different waste water treatment plants in Romania. All three HBCD isomers were detected in all 6 tested WWTP sludge samples (table 4).

The abundance of α-HBCD and γ-HBCD in the analyzed samples is very close, the detected concentrations being almost similar. In three samples, S1, S3 and S4, α-HBCD concentration values were slightly higher than those detected for γ-HBCD: 42.7, 7.8, 44.0 ng/g dw, compared to 38.0, 7.1 and 39.7 ng/g dw. On the contrary, the concentration values detected from S2, S5 and S6 samples were higher for γ-HBCD, 75.8, 39.7 and 54.5 ng/g dw, than for the ones detected for α-HBCD, 63.5, 37.8 and 38.5 ng/g dw. Although the γ -HBCD isomer is predominant in commercial formulations (containing 3–30% of α- and β-HBCD and 70–95% of γ-HBCD) and has higher hydrophobicity than α- and β-HBCD, being inclined to be the most accumulated in sewage sludge [19], concentrations range determined for the two isomers - α-HBCD and γ-HBCD in real samples were quite close. A reasonable explanation for these levels might be isomer inter-conversion phenomena from γ-HBCD to α-HBCD, influenced by photochemical or thermodynamic processes [20]. Concerning β-HBCD, this compound was detected at the lowest levels among the three isomers, between 5.6 and 14.7 ng/g dw, which is consistent with data presented by other published studies.

Conclusions

A rapid, accurate and robust LC-ESI(-)MS/MS method was developed and validated to identify and determine three HBCD diastereomers in WWTP sewage sludge samples. Chromatographic conditions (including mobile phase, column and modifier) and MS/MS parameters were optimized for complete isomers separation and high MS detection sensitivity to be able to determine trace amounts of these contaminants in a very complex matrix like WWTP sewage sludge. In sewage sludge samples, recovery efficiencies were in the range of 79 ÷ 86% with intra-day and inter-day precision better than 9.3% and 14.5%. All three HBCD isomers were detected in 6 tested WWTP sludge samples from Romania, α- and γ-HBCD being the detected in the highest amounts. The overall method quantitation limits (LOQ) for the three Hexabromocyclododecane isomers were situated between 0.7 and 1.4 ng/g, much lower than those found in other studies for similar methods (ultrasonic extraction followed by LC-MS/MS analysis) [21, 22]. The concentrations found for the α-HBCD, β-HBCD and γ-HBCD were situated in the ranges 7.8 - 63.5 ng/g, 3.0 - 14.7 ng/g and 7.1 - 75.8 ng/g dry weight, respectively. α-HBCD concentration levels are generally similar to those obtained for γ-HBCD which is not consistent with the percentages supplied in the HBCD technical mixtures commercial products. This indicates that γ-HBCD suffers partial isomer inter-conversion to α-HBCD which is the more thermodynamically stable form. Also, different degradation rates for these two isomers may be another plausible explanation.

Acknowledgements. The authors acknowledge the financial support offered by The National Research Program "Nucleu" through Project PN 16-25.01.10.

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Manuscript received: 15.01.2017