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Ultra high performance liquid chromatography–time-of-flight high resolution mass spectrometry in the analysis of hexabromocyclododecane diastereomers: Method development and comparative evaluation versus ultra high performance liquid chromatography coupled to Orbitrap high resolution mass spectrometry and triple quadrupole tandem mass spectrometry

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ABSTRACT

An efficient ultra high performance liquid chromatography (UHPLC)–time-of-flight high resolution mass spectrometry (TOF-HRMS) method was elaborated for the determination of hexabromocyclododecane (HBCD) diastereomers in fish samples and compared against UHPLC–Orbitrap-HRMS and UHPLC–triple quadrupole (QqQ) tandem MS (MS/MS) techniques. The TOF-HRMS analyzer was operated at high resolution (>10 000 full width at half maximum (FWHM)) with scanning the m/z range from 600 to 700, to achieve picogram quantitation limits. The effects of various operational parameters on the instrumental response were systematically investigated. Evaluation of the influence of sample clean-up procedure steps on signal suppression effect including removal of the matrix components by means of destructive acidic treatment or non-destructive gel permeation chromatography (GPC), and additional Florisil column chromatography step showed that the analytical response of UHPLC–TOF-HRMS system is much more affected by the presence of matrix components in the final extracts in comparison with UHPLC–Orbitrap-HRMS and UHPLC–QqQ-MS/MS systems. The method was robustly validated and used for the analysis of eel (*Anquilla anquilla*) samples originating from a Latvian lake. UHPLC–TOF-HRMS showed a suitable performance under the optimized conditions: recoveries for three selected diastereomers in the range of 99–116%; repeatability and intermediate precision expressed as relative standard deviation (RSD) in the ranges of 2.3–7.1% and 2.9–8.1%, respectively. The elaborated method achieved instrumental limits of quantification (i-LOQ) of 0.9–4.5 pg on column that were suitable for the trace analysis of three HBCD diastereomers, corresponding to the method limits of quantification (m-LOQ) of 7.0–29 pg g^{-1} wet weight (w.w.). The efficiency of UHPLC–TOF-HRMS method was evaluated by comparing the performance characteristics and analytical data from real samples with the validation data and real sample results obtained by applying UHPLC–Orbitrap-HRMS and UHPLC–QqQ-MS/MS techniques for the analysis of HBCD in the same fish samples. Statistical assessment of the experimental data by means of the Fiedman's test revealed that UHPLC–TOF-HRMS, UHPLC–QqQ-MS/MS and UHPLC–Orbitrap-HRMS techniques produced adequate and similar results regarding the HBCD content in fish samples. The presence of HBCD diastereomers was confirmed in all the analyzed eels at concentrations up to 554 pg g^{-1} w.w. for total HBCD and a diastereomer pattern typical for aquatic biota was observed with strong predominance of α-HBCD. The UHPLC–TOF-HRMS is an appropriate technique for diastereomer-specific quantification of HBCD content in fish samples.

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1. Introduction

Brominated flame retardants (BFRs) nowadays are ubiquitous contaminants due to their widespread usage in the manufacturing of plastics, textiles, paints, and other consumer materials. The addition of these compounds to combustible synthetic materials provides the protection from ignition or hinders the spread of fire [1]. Among the class of BFRs, HBCD is one of the essential products with 6000 tonnes of annual usage in Europe [2]. HBCD is an additive flame retardant and discarding of the material containing HBCD results in the release of this chemical to the environment. Technical HBCD mixtures contain mainly α-, β-, and γ-HBCD diastereomers with the latter being predominant [3]. High persistence, low water solubility and high octanol–water partition coefficient ($\log K_{ow}$) of the HBCD are similar to those for persistent organic pollutants (POPs), therefore it was classified in 2008 by the European Commission (EC) as a bioaccumulative and toxic compound [4]. Since the HBCD was ubiquitously detected in aquatic biota and environmental objects [5,6], several scientific papers were devoted to the analytical problems and occurrence of this contaminant [7]. The most extensively used methods for the analysis of HBCD in environmental samples are based on gas chromatography (GC) or LC coupled to mass spectrometry (MS) [8]. However, due to the thermal lability and interconversion of HBCD under the conditions of GC, only LC based methods could provide the diastereomer-specific data on the occurrence of this contaminant [8,9]. The diastereomer-specific identification and quantification of HBCD becomes of great importance due to the dissimilarities in the structure of α-, β-, and γ-HBCDs and hence the differences in bioaccumulation, biotransformation and persistence. Moreover, the isomeric pattern could differ among the analyzed matrices or for particular production conditions, therefore “fingerprint” data for the cases of observed bioaccumulation or contamination may be obtained.

Mass spectrometric techniques are the methods of choice in analysis of POPs, since they provide the necessary selectivity and sensitivity for the determination of these contaminants at background levels [10]. Among the different MS instrumentation, a unit resolution MS techniques coupled to LC are typically used for the detection of HBCD [7]. Due to the versatility and relatively low cost, QqQ based LC–MS systems are used most frequently in the detection and quantification of HBCD. Several MS/MS methods utilizing selective reaction monitoring (SRM) detection mode were developed for various types of matrices including sewage sludge [11], sediments [12], indoor and airborne dust [13,14], food products [15], and fish [16–19]. Monitoring of the specific transition $[M-H]^-$ (m/z 640.6) → $[Br]^-$ (m/z 78.9 and 80.9) provides a selective analysis of HBCD at trace levels.

Recent developments in the field of LC–MS enable improved detection and quantification of harmful substances by application of HRMS, providing more accurate and more precise analysis of contaminants in complex samples [20]. Among the HRMS based approaches for residue trace analysis, application of TOF-HRMS and Orbitrap-HRMS coupled to HPLC or UHPLC has become more affordable [20]. The usage of LC–TOF-HRMS methods can be found in a variety of analytical applications, although only scarce mentions regarding the analytical possibilities of this approach in the analysis of BFRs is provided. By our knowledge, at the present time only one published study report has been devoted to using an LC–Q-TOF-HRMS method operated in selected ion monitoring (SIM) mode for the analysis of HBCD in toxicological studies, without describing the essential performance characteristics of the method [21]. The LC–TOF-HRMS techniques operated in scan mode are advanced and efficient, providing the possibility for the fast screening of target analytes and performance characteristics, such as sensitivity, linearity, and reproducibility comparable to LC–MS/MS [20]. The high resolution and fast scanning speed of TOF-HRMS analyzer offers particular advantages in the terms of operation in scan modes over the wide range of m/z values. The analytical performance of Orbitrap-HRMS can support a wide range of applications including the analysis of trace-level components in complex mixtures or detection of food and feed contaminants [22]. Recent developments show the great perspective of the analytical possibilities of LC–Orbitrap-HRMS technique in diastereomer-specific HBCD analysis of fish samples [23]. The powerful advantages of Orbitrap-HRMS, such as high mass resolution and mass accuracy up to 1 ppm result in high selectivity for target analytes and is of great importance in advanced identification of emerging contaminants, such as POPs in complex matrices. Combination of high resolution (up to 300 000 FWHM) with a fast scan speed and multiplexing capabilities provides new perspectives in the development of confirmatory analytical methods for the determination of ultra-trace levels of dangerous substances, such as BFRs.

At the present time, there is no information on the application of the TOF-HRMS methods for analysis of HBCD in the scan mode, and therefore this field of mass spectrometric detection could be a versatile tool in the analysis of BFRs in terms of elaboration of high throughput methods. Contrary to Q-TOF-HRMS technique, for which scanning speed is quite limited by the quadrupole (<20 scans s^{-1}) due to fact that only limited voltage ramp could be applied to the ring electrodes, in TOF-HRMS analyzer all ions are detected within a very short period and a complete acquisition of the mass spectrum is limited only by the flight time of the higher mass, thus TOF-HRMS technique has much higher spectral acquisition rates. Fast scanning speed and high resolution of the TOF-HRMS technique could enable great achievements in the field of ultra-trace analysis of BFRs, especially when UHPLC is utilized, thus providing additional advances in the elaboration of high throughput methods.

Within the framework of this study, UHPLC–TOF-HRMS based analytical methodology for the diastereoselective determination of HBCD is presented. The provisionally optimized clean-up procedure and UHPLC conditions were previously used for successful sample treatment and chromatographic separation of the analytes of interest [23]. In order to provide a more complete information regarding the analytical capabilities of the elaborated method, the influence of the experimental conditions and importance of sample clean-up stages in the detection of HBCD were evaluated. Moreover, taking into account the absence of comparative evaluation of the most frequently used LC–MS based HBCD detection techniques, we additionally report a detailed information on the analytical capabilities of UHPLC–Orbitrap-HRMS and UHPLC–QqQ-MS/MS systems in the analysis of this contaminant in fish samples, including the influence of sample clean-up steps on the response of the employed MS systems. To compare the examined LC–MS techniques for the analysis of HBCD, the previously mentioned clean-up and UHPLC conditions [23] were kept constant and three types of MS systems–TOF-HRMS, Orbitrap-HRMS, and QqQ-MS/MS were applied for the detection of this BFR in fish. The elaborated analytical methods were robustly validated and used for the analysis of eel samples collected in Latvia, and these results were compared. The obtained data show that a TOF-HRMS system could provide a similar performance to the typically used MS/MS systems.

2. Experimental

2.1. Chemicals and materials

Standard solutions of the individual native and $^{13}C_{12}$-labelled α-, β-, and γ-HBCD diastereomers were purchased from Cambridge Isotope Laboratories, Inc. (Andover, USA) and AccuStandard, Inc. (New Haven, USA) at the concentrations of 50 and 100 $\mu g\,mL^{-1}$ in toluene. HPLC grade methanol, pesticide grade

n-hexane, dichloromethane, ethyl acetate, and cyclohexane were purchased from Lab-Scan (Glivice, Poland). Silica gel and Florisil were from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Sulphuric acid and sodium sulphate were obtained from Acros (New Jersey, USA). High-purity water (18.2 MΩ) was prepared using Millipore Milli-Q purification system (Billerica, MA, USA). Calibration solutions were prepared by serial dilution of stock solutions in toluene, evaporation of the organic solvent to dryness under gentle nitrogen stream and redissolving the residue in methanol.

2.2. Sample collection and storage

Eight eel (*Anquilla anquilla*) specimens of various age, length, and weight were caught in the lake Usma in the north-west part of Latvia in August 2013. After individual laboratory codes were given, the length and weight of all specimens was measured. The average length and weight of eels was 55 cm (range from 50 to 67 cm) and 1.00 kg (range from 0.82 to 1.56 kg). During the sample pretreatment the specimens were dissected, the fillets were isolated and homogenized using a food blender (Kenwood FP101T, Kenwood Ltd, UK), and the homogenates were packed into polyethylene bags and stored at −18 °C until analysis.

2.3. Sample extraction and clean-up steps for the evaluation of influence on the instrumental response

To prevent the degradation of potentially photolabile HBCDs, sample extraction, clean-up, and all handling procedures were performed using amber-coloured glassware or by wrapping the glassware in aluminium foil. The samples for validation study and the analyzed eel samples were prepared according to the analytical scheme reported in our previous study, including extraction with organic solvents, destructive treatment of the extracts with concentrated sulphuric acid and acid impregnated silica gel, and further clean-up by column chromatography on Florisil [23]. For the validation purposes and for establishing the influence of different sample clean-up steps on the instrument response to the analytes of interest, a butter fish homogenate was used as model matrix and was treated according to the procedures given below. The model matrix was previously analyzed for the content of HBCD diastereomers and found to contain only traces of α-HBCD (triplicate analysis of the material showed the average concentration of 11.0 pg g^{-1} sample fresh weight (f.w.)) whereas β- and γ-HBCDs were found to be below the LOQ. To evaluate the possible instrumental signal suppression by the matrix components, the sample extracts obtained after the corresponding clean-up stage were spiked with the appropriate volume of HBCD diastereomer solution in toluene and were evaporated until dryness under a gentle stream of nitrogen. The obtained samples were reconstituted in 200 μL of methanol–water–acetonitrile mixture (60:20:20, v/v/v), and transferred to an autosampler vial. The amount of the standard solution added before the instrumental analysis provided the final 10 pg μL^{-1} concentration for each HBCD stereoisomer. The samples for instrumental signal suppression experiments were prepared in triplicate ($n = 3$) and each sample was injected in duplicate. It was found that the RSD for the obtained instrumental responses for each set of samples corresponding to the evaluated sample preparation step or their combinations was below 20%.

2.3.1. Sample extraction

A 5.0 g aliquot of homogenized fish sample was mixed with 30 g of anhydrous sodium sulphate and, in the case of validation study or analysis of real samples, a 40 μL portion of $^{13}C_{12}$-labelled α-, β-, and γ-HBCD diastereomer solution in toluene (100 pg μL^{-1}) was added. After the equilibration for at least 1 h, the sample was extracted with 40 mL of dichloromethane/*n*-hexane (1:1, v/v) by shaking for 20 min. The organic extract was filtered through a layer of sodium sulphate and the matrix homogenate was washed with additional 30 mL of the extraction solvent mixture. The organic extracts were combined and treated according to the procedures described in the further sections.

2.3.2. Acidic treatment of the sample extracts

In the case of acidic clean-up, the sample extracts obtained after the sample extraction procedure were treated with 10 mL of 37 N sulphuric acid by intense shaking for 15 min. The mixture was allowed to stand for 15 min and centrifuged at 3000 rpm using a Falcon 6/300 benchtop centrifuge (MSE, London, UK). The bottom (aqueous) layer was discarded and the organic extract was treated with 10 g of silica gel impregnated with sulphuric acid (50% of 37 N sulphuric acid). After intense shaking of the mixture, the organic phase was filtered and the silica residue was washed with 20 mL of the extraction solvent mixture. The filtrate and washings were combined and transferred to a round-bottom flask, and in the case of further clean-up concentrated to approximately 1 mL on a rotary evaporator, and the solvent was exchanged to 1 mL of *n*-hexane. In the case of using only the aforementioned clean-up step without any other treatment of the sample, the organic extracts were spiked with standard solution of HBCD, evaporated to dryness under a gentle stream of nitrogen and reconstituted in methanol–water–acetonitrile mixture as described in Section 2.3.

2.3.3. Gel permeation chromatography (GPC)

In the case of using GPC, the organic extracts obtained after the sample extraction procedure were transferred to a round-bottom flask and evaporated to fatty residue. The fat fraction was quantitatively transferred to 16 mL GPC autosampler vials and diluted with the mobile phase to 5.0 mL. The sample extracts were injected into LC Tech Freestyle™ GPC system (Dorfen, Germany) consisting of an HPLC pump, autosampler and a fraction collector, and high molecular weight substances were removed on a glass column (50 cm × 2.5 cm) filled with 50 g of Bio-Beads S-X3 (Bio-Rad, Philadelphia, USA) stationary phase with cyclohexane/ethyl acetate (1:1, v/v) mobile phase at a flow rate of 5 mL min^{-1}. The following automated GPC programme was used: dump time 0–19 min, collection time 19–45 min. The collected fractions were transferred to round-bottom flasks and in the case of further clean-up concentrated to approximately 1 mL on a rotary evaporator, and the solvent was exchanged to 1 mL of *n*-hexane. In the case of using only the aforementioned clean-up step without any other treatment of the sample, the organic extracts were spiked with a standard solution of HBCD, evaporated until dryness under a gentle stream of nitrogen and reconstituted in methanol–water–acetonitrile mixture as described in Section 2.3.

2.3.4. Florisil column chromatography

In the case of using the additional clean-up step of a Florisil column, the extracts obtained from the acidic treatment or GPC clean-up steps were further treated on a glass column (25 cm × 1.0 cm) filled with 3.0 g of Florisil deactivated with 3% water: after the addition of the sample extract, the column was washed with 20 mL of *n*-hexane, and the eluate was discarded. The analytes of interest were eluted from the column with 40 mL of *n*-hexane/dichloromethane (1:1, v/v) mixture, the fraction was concentrated by rotary evaporation and reconstituted in methanol–water–acetonitrile mixture as described in Section 2.3.

2.4. Instrumental analysis

2.4.1. UHPLC separation of the target analytes

Within the framework of this study, optimized chromatography conditions were kept constant for all the applied instrumental

UHPLC–MS methods. UHPLC separation of target compounds was carried out using Kinetex C_{18}, 100 mm × 2.1 mm, 1.7 μm reversed-phase analytical column at 25 °C, applying a flow rate of 250 μL min^{-1} with a mobile phase gradient based on (A) methanol–water (75:25, v/v) and (B) acetonitrile. The effective 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 column was equilibrated for 2.0 min between the runs. The injection volume of 10 μL was used both for the standard solutions and sample extracts.

2.4.2. Mass spectrometry analysis

During the tuning procedure, the signal of HBCD diastereomers was preliminarily optimized for the highest response of the $[M-H]^-$ ion and, in the case of using the MS/MS option, the system was optimized to obtain the highest response for the specific SRM transitions. Specific transitions selected for HBCD were based on the transition $[M-H]^- \rightarrow [Br]^-$. During the infusion experiments, direct introduction of the target compounds (native and $^{13}C_{12}$-labelled α-, β-, and γ-HBCDs, 1 ng μL^{-1} of each in methanol) into the ESI$^-$ interfaces of the MS systems was performed at 10 μL min^{-1}. For the introduction-through-the-column (ITC) experiments, injection of a standard solution of HBCD diastereomers and the corresponding internal standards (10 pg μL^{-1} of each in mobile phase) into the MS system was performed through the LC column and the gradient was run according to Section 2.4.1.

2.4.2.1. The UHPLC–TOF-HRMS system. The UHPLC–TOF-HRMS instrument consisted of an Agilent Technologies 1290 Infinity UHPLC system coupled to a 6230 TOF mass spectrometer (Santa Clara, CA, USA) equipped with a heated electrospray ionization interface. The negative ion mode was used for the acquisition of mass spectra. A TOF-HRMS detection in the scan mode over the m/z range 600–700 was used for a quantitative determination of selected compounds using the two most abundant $[M-H]^-$ ions of the respective molecular ion cluster for both the native and the $^{13}C_{12}$-labelled surrogates. The nominal channels monitored for HBCD diastereomers 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 $^{13}C_{12}$-labelled surrogates. External calibration of the TOF-HRMS instrument was performed before each batch of samples over the mass range of m/z 100–3200 and achieving mass resolving power greater than 10 000 FWHM according to the guidelines provided by the instrument supplier. For the raw data treatment and targeting/quantitation of selected contaminants, Agilent Technologies MassHunter Workstation software was used. The details of the optimized instrumental conditions are summarized in Table 1.

2.4.2.2. The UHPLC–Orbitrap-HRMS system. UHPLC–Orbitrap-HRMS analyses were performed on a Thermo Accela UHPLC system (Zwingen, Switzerland) coupled to an Orbitrap Q Exactive mass spectrometer (Bremen, Germany) equipped with heated electrospray ionization (HESI-II) interface operated in the negative mode. Data were obtained while operating in the targeted-SIM (t-SIM) mode using the same m/z fragments selected for the TOF-HRMS technique. External calibration of the Orbitrap-HRMS system was performed before each batch of samples over the mass range of m/z 50–2000 according to the guidelines provided by the instrument supplier. ThermoXcalibur™ and TraceFinder™ 3.0 software were used for the raw data interpretation and for the targeting/quantitation of selected contaminants. The details of the optimized instrumental conditions are summarized in Table 1.

2.4.2.3. The UHPLC–QqQ-MS/MS system. For MS/MS analyses an AB SciexQTrap 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with heated electrospray ionization interface and a Waters Acquity UHPLC system (Waters, Milford, MA, USA) were used. The data acquisition was performed in the negative SRM mode to obtain sufficient number of quantification points for the confirmation of each HBCD diastereomer. Analyst® software was used to control all the components of the instrument and for the data acquisition and processing. The channels monitored for the three HBCD diastereomers were m/z 640.6 → 78.9 (quantification) and m/z 640.3 → 80.9 (confirmation), and the internal standard channels were m/z 652.6 → 78.9 and m/z 652.6 → 80.9. External calibration of the mass spectrometer was performed according to the manufacturer requirements. Detailed instrumental conditions of the system are summarized in Table 1.

2.5. Quality assurance/quality control (QA/QC) in the analysis of real samples

The identification criteria for the analytes of interest were based on the retention times of native components and $^{13}C_{12}$-labelled internal standards, and the isotopic peak ratios of the monitored ions or SRM transitions. The acceptable deviation of the isotope ratio of two monitored ions or SRM transitions (target/confirmation) was below 15% of the value obtained for the medium calibration point. A five-point calibration curve was checked with the relative response factors (RRFs) over the concentration range of 1.00–100 pg μL^{-1} and was used for the quantitation of HBCD diastereomers in each sample run. Procedural blanks consisting of anhydrous sodium sulphate aliquot typically used for routine samples were taken through all steps of analytical procedure and analyzed in each sample sequence. Procedural blanks were found to be free from the analytes of interest. Quantitation of analytes of interest was based on the stable isotope dilution with the $^{13}C_{12}$-labelled surrogates and internal standardization.

3. Results and discussion

3.1. Optimization of Orbitrap-HRMS and QqQ-MS/MS conditions

Analytical methods for the determination of HBCD diastereomers were based on using the conventional QqQ-MS/MS techniques reported in many earlier studies [7,8]. The mass spectrometry conditions for the QqQ-MS/MS system were established taking into account the previously proposed procedures. The precursor-product ion transitions and optimized collision energies are summarized in Table 1. Optimized instrumental conditions of Orbitrap-HRMS system were used according to the results of our previous study [23] and are presented in Table 1 for comparative purposes.

3.2. The optimization of TOF-HRMS conditions

An extensive method development was performed in order to establish the optimal sample clean-up and instrumental conditions taking into account the requirements for final extract purity and the equipment capabilities. Once the sample extraction and clean-up procedures and chromatographic conditions were optimized to provide satisfactory recoveries of the analytes of interest, tolerable final extract purity and acceptable chromatographic separation of three HBCD diastereomers [23], the detailed optimization of the essential instrumental parameters of TOF-HRMS system was performed. Preliminary investigations were carried out in order to establish the parameters of the TOF-HRMS instrument able to provide the highest response for the analyzed compounds under the used chromatographic conditions. For this purpose, ITC analysis

Table 1
The optimized instrumental conditions for the determination of HBCD diastereomers using different MS techniques.

	Orbitrap-HRMS	TOF-HRMS	QqQ-MS/MS
ESI⁻ conditions			
Drying gas temperature	–	260 °C	–
Drying gas flow	–	13 L min^{-1}	–
Nebulizer pressure	–	25 psig	–
Sheath gas temperature	–	280 °C	–
Capillary voltage	–	3.0 kV	–
Nozzle voltage	–	3.0 kV	–
Fragmentor voltage	–	150 V	–
Skimmer voltage	–	75 V	–
Sheath gas flow/pressure	15 a.u.*	12 L min^{-1}	60 psi
Auxiliary gas flow/pressure	5 a.u.*	–	30 psi
Capillary temperature	250 °C	–	–
Source heater temperature	250 °C	–	400 °C
Spray voltage	4.5 kV	–	4.5 kV
S-lens radio frequency	50	–	–
MS conditions			
Maximum injection time	100 ms	–	–
Automatic gain control (AGC target)	5×10^4	–	–
MS resolution	35 000 FWHM	15 000 FWHM	UNIT
Detection mode	t-SIM	FULL SCAN	SRM
Declustering potential	–	–	90 V
Collision energy	–	–	60 V

a.u.*—arbitrary units.

of the standard solution of HBCD diastereomers and the corresponding $^{13}C_{12}$-labelled surrogates in mobile phase (10 pg μL^{-1}) through the LC column was performed under the optimized gradient, and the instrumental parameters of the TOF-HRMS system were investigated in order to obtain the highest signal-to-noise (S/N) ratios for the chromatographic peaks of the analyzed compounds.

As it was shown in earlier studies devoted to LC–MS based analytical methods for the determination of HBCD, the ion at m/z 640.7 is typically predominant in the ESI⁻ spectra of HBCD, corresponding to the [M−H]⁻ fragment [9,16,19,23]. For the UHPLC–TOF-HRMS system used in the current study, the predominance of the [M−H]⁻ fragment at m/z 640.6514 was confirmed, although the presence of a weaker signal corresponding to [M+Cl]⁻ at m/z 676.6290 was apparent as well. Some studies reported accuracy problems in the analysis of HBCD due to the presence of this interfering signal [9,24]. For its suppression or control, addition of ammonium acetate to the mobile phase was proposed [9,25,26], but this approach does not completely eliminate the presence of [M+Cl]⁻ fragment, and generates an additional acetate adduct with HBCD molecules [27]. In the current study, the presence of the [M+Cl]⁻ fragment varied during the validation experiments and the application of the method to real samples, although the intensity of this signal did not exceed 15% of the predominant [M−H]⁻ fragment. Moreover, taking into account the isotope-dilution internal standardization approach used for quantitation purposes, the influence of the [M+Cl]⁻ fragment formation during the instrumental analysis seems to be insignificant in terms of accuracy and precision. Fig. 1 shows TOF-HRMS spectra of the chromatographic peak of α-HBCD, which is typically predominant in biota, and the corresponding $^{13}C_{12}$-labelled internal standard, obtained in full scan mode within the m/z range from 600 to 700.

As it was expected, due to the thermolabile properties of HBCD, the foremost parameter of the system was the ionization chamber temperature. The maximum ion signal was obtained at the optimum temperatures of 260 °C and 280 °C for the drying gas and sheath gas, respectively. Although there was no significant effect from the drying and sheath gas flows inside the ESI source, a slight increase of the S/N ratios was observed at the maximum possible velocities of the gases. The influence of the nebulizer pressure was investigated in the range of 20–55 psig. The optimal pressure region was found to be in the range from 20 to 35 psig, with a sharp drop of sensitivity at pressures above 40 psig.

Another key parameter in terms of instrument sensitivity is the fragmentor voltage (identical to the term “cone voltage” in the instruments of other manufacturers). The fragmentor voltage influences ion transmission and in-source fragmentation. Thus, at higher fragmentor voltage settings collision induced dissociation (CID) can be initiated in the region between the end of the transfer capillary and the first skimmer cone, so that fragmentation increases [28]. In the current study the optimal potential of the fragmentor was set at 150 V. The effects of the capillary and nozzle voltages were not apparently expressed; better S/N ratios were observed at the moderate potential of 3000 V for both options, however. The voltage of 75 V applied to the skimmer and electrostatic lens (encountered by the ions after exiting the capillary) was found to be optimal for achieving a better sensitivity of the system. An obvious drop of sensitivity was observed below the skimmer voltage of 45 V and above 75 V. The optimized instrumental conditions of the UHPLC–TOF-HRMS instrument are outlined in Table 1.

3.3. The importance of sample clean-up stages on the signal suppression effect in the analysis of HBCD

One of the most important factors that can affect the performance of the MS system is the signal suppression caused by matrix components. Several papers in the field of LC–MS are devoted on this effect, and it could be seen from the literature that MS systems coupled to ESI are more vulnerable to signal suppression in comparison to atmospheric-pressure chemical ionization (APCI) or atmospheric-pressure photo-ionization (APPI) techniques [29–31]. The signal suppression effect depends mostly on the efficiency of ionization interface of the LC–MS system and is not connected to the type of MS analyzer. The possible reasons of the signal suppression are: (1) the charge competition between the analyte and signal suppressing substances, resulting in a decreased conductivity of the liquid phase and reduced ionization of the analyte; (2) the reduction of the droplet evaporation efficiency due to the increasing surface tension and viscosity of the liquid phase in the presence of large amounts of signal suppressing substances; (3) the reduction of the ionization efficiency due to the reactions in

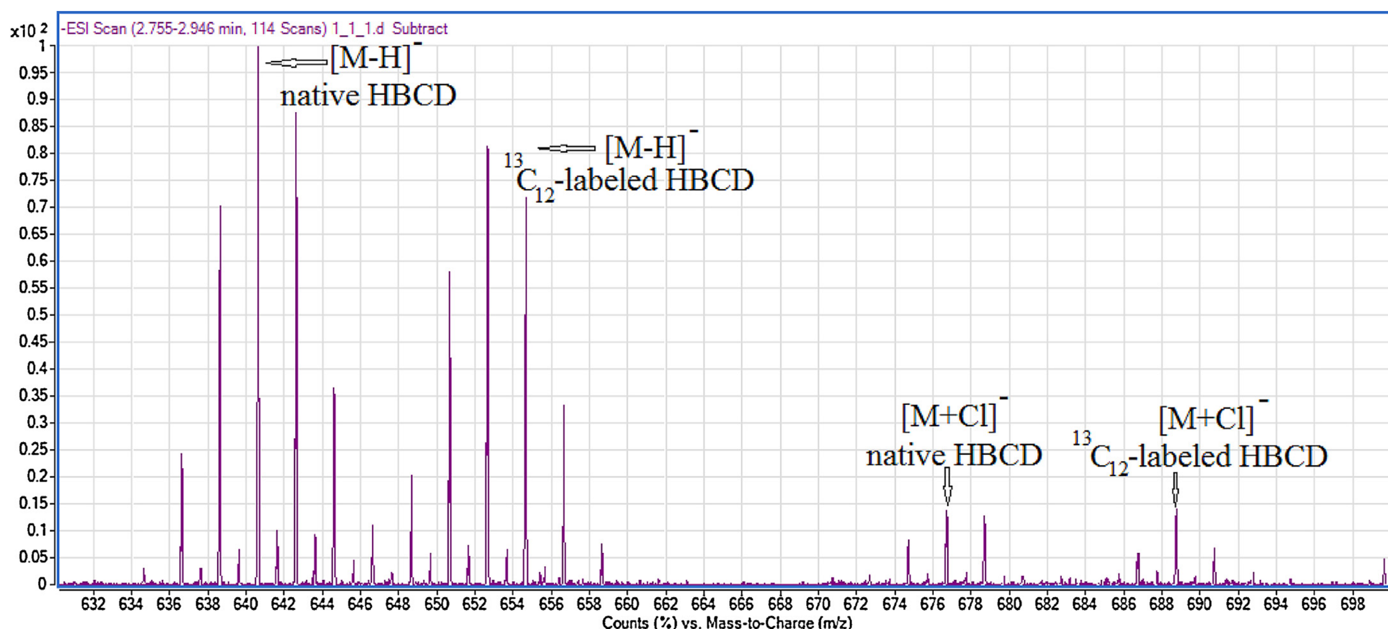

Fig. 1. The TOF-HRMS spectra of the chromatographic peak corresponding to native α-HBCD and $^{13}C_{12}$-labelled α-HBCD (UHPLC–TOF-HRMS analysis after the injection of 100 pg of each compound on the column).

gas phase between the analyte and signal suppressing molecules [29,30]. Due to the complexity of the food and environmental matrices, the primary concern of the signal suppression phenomenon in LC–MS is the severe decrease of the sensitivity of the method. An investigation of the importance of proposed clean-up steps was carried out by evaluation of the signal suppression for three LC–MS techniques. The influence of the signal suppression effect was estimated by adding known amounts of analytes of interest to the final sample extracts, which were processed using different clean-up steps (or their combinations). The influence of the sample weight taken for analysis was evaluated as well. The S/N ratios, which were calculated for the chromatographic peaks of HBCD diastereomers obtained for the investigated extracts, were compared to those obtained for standard solution of equivalent analyte concentrations in the mobile phase. The data for the analyte solution in mobile phase provide a relative 100% response (S/N) value, whereas the data for the same amount of compound added to processed samples show the effect of sample matrix on MS response and on the obtained S/N. Fig. 2 shows decreasing S/N values obtained by the investigated LC–MS techniques for each HBCD diastereomer, by application of different clean-up steps or their combinations and using different sample amounts for analysis.

Firstly, the efficiency of destructive (acidic treatment) and non-destructive (GPC) approaches for removing matrix components was evaluated. Both methods and their combinations are well known in the sample preparation for POP analysis (particularly for HBCD [8]). Acidic treatment was chosen as one of the most efficient and simplest ways to remove high molecular matrix components (e.g., lipids) from the sample extract. However, it should be noted that such destructive approach could be used only when the stability of the compounds of interest under the harsh conditions of acidic treatment is assured. On the contrary, GPC provides a non-destructive procedure for high molecular compound removal, which is based on the separation of the sample components according to molecular size, and this type of chromatography offers great advantages in the sample preparation for potentially labile compounds, particularly for non-targeted analysis [32]. Nevertheless, the efficiency of the separation of high molecular matrix components is usually not more than 95%, therefore additional clean-up steps are needed in some cases. The results of our study indicate that significant signal suppression effects could be observed in the analysis of HBCD using the examined LC–MS methods. The UHPLC–TOF-HRMS system seems to be most influenced by the signal suppression in comparison with UHPLC–QqQ-MS/MS and UHPLC–Orbitrap-HRMS. Application of the one-stage clean-up protocol including only acidic treatment of the sample extract or GPC caused a more than 90% sensitivity drop for the UHPLC–TOF-HRMS system, while the analytical response of the UHPLC–QqQ-MS/MS and UHPLC–Orbitrap-HRMS systems was suppressed by about 50%. No significant differences between these analytical techniques in terms of signal suppression were obtained neither with destructive nor non-destructive clean-up.

A significant improvement of S/N values was achieved by implementing an additional adsorption chromatography clean-up stage on a Florisil column. For UHPLC–TOF-HRMS system the S/N ratio for chromatographic peaks due to analytes of interest could reach up to 50% of the instrumental response obtained for standard solution. For the UHPLC–IT-MS/MS and UHPLC–Orbitrap-HRMS systems, the application of a two-stage clean-up protocol including removal of high molecular substances and Florisil column chromatography could provide S/N ratios up to 80–90% of the ratios obtained for standard solution. Florisil column chromatography provides better sensitivity of analysis because the signal suppressors originating from the matrix could be either fractionated or permanently adsorbed during this clean-up stage. Moreover, taking into account the fact that fish samples usually contain significant amounts of organobromines (e.g. polybrominated diphenyl ethers (PBDEs)) which could potentially interfere with the mass spectrometric analysis of HBCD by providing similar mass fragments during the ionization of the sample extract, the using of Florisil column could ensure an additional benefit due to the ability to isolate these potential mass interferents in separate fractions.

In addition to evaluating the importance of the proposed clean-up steps or their combinations, the influence of the sample amount taken for analysis was investigated for aliquots of 5, 10, and 15 g of butter fish homogenate. The observed degree of signal suppression indicates that this effect plays a significant role, and it was more

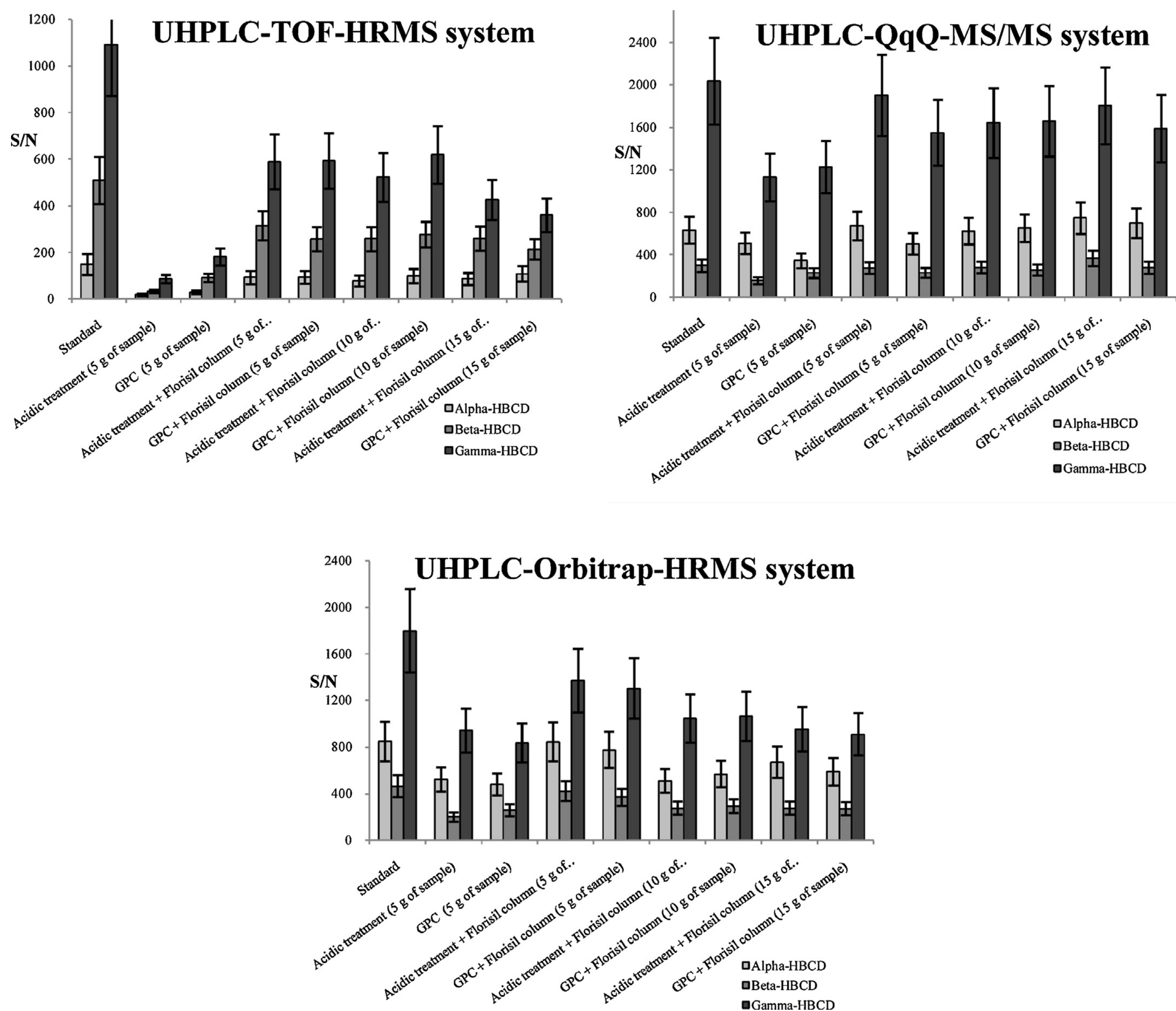

Fig. 2. Changes of the S/N values obtained for the investigated LC–MS techniques for each HBCD diastereomer by application of different clean-up steps or their combinations and different sample amounts taken for analysis.

expressed for UHPLC–TOF-HRMS and UHPLC–Orbitrap-HRMS systems, while the UHPLC–QqQ-MS/MS system seems to be more robust in terms of this phenomenon (Fig. 2).

3.4. Comparison of performance characteristics of the applied methods

Since a target-oriented approach was used for the determination of HBCD diastereomers, destructive acidic treatment clean-up protocol with additional purification stage on a Florisil column was chosen for validation exercises as the simplest and less time consuming in comparison with a GPC based clean-up procedure. Essential analytical characteristics such as linearity, accuracy (recovery), repeatability (intra-day precision), intermediate precision (inter-day precision), i-LOQ and m-LOQ were examined in order to evaluate the analytical performance of the compared analytical procedures. The methods were provisionally validated using butter fish homogenate, and the performance of the methods was evaluated by run-to-run ($n = 5$) and day-to-day ($n = 3$) analyses of spiked matrix at three concentration levels (200, 1000, and 2000 pg g^{-1} w.w. of each HBCD diastereomer). The repeatability and intermediate precision were expressed as RSD from the results obtained during the recovery experiments. Table 2 outlines the above mentioned analytical performance parameters for the three applied MS systems.

During the linearity experiments, both the matrix matched and solvent matched calibration experiments were performed at five calibration levels from 1.00 to 100 pg μL^{-1}, and each calibration solution was analyzed in triplicate. The working range was selected by taking into account the typical distribution profiles (e.g., strong predominance of α-HBCD in comparison to β-and γ-HBCDs in biota samples and predominance of the γ-HBCD for environmental objects), and the levels of HBCD diastereomers in the most frequently analyzed objects, as well as sample intake and the amount of analyte injected on-column limited by the final volume and injection volume of the sample extract. For the Orbitrap-HRMS and QqQ-MS/MS instruments, the equations of the calibration curves were fitted to a linear function and the relationship obtained by internal standard method was found to be rectilinear with

Table 2
Analytical characteristics of the compared LC–MS techniques for analysis of HBCD diastereomers.

Compound	Linearity of measurement, pg μL^{-1}	UHPLC–QqQ-MS/MS			UHPLC–Orbitrap-MS			UHPLC–TOF-MS		
		Detection traces	i-LOQ, pg	m-LOQ, pg g^{-1} f.w.	Detection traces	i-LOQ, pg	m-LOQ, pg g^{-1} f.w.	Detection traces	i-LOQ, pg	m-LOQ, pg g^{-1} f.w.
α-HBCD	1.00–100	640.6 → 78.9/80.9	1.3	5.0	638.6396/640.6374	1.1	4.0	638.6396/640.6374	4.5	29
β-HBCD	1.00–100	640.6 → 78.9/80.9	2.1	9.0	638.6396/640.6374	3.0	13	638.6396/640.6374	1.4	9.0
γ-HBCD	1.00–100	640.6 → 78.9/80.9	0.9	4.0	638.6396/640.6374	1.4	7.0	638.6396/640.6374	0.9	7.0

1st validation level

Compound	Spiking level, pg g^{-1} f.w.	UHPLC–QqQ-MS/MS			UHPLC–Orbitrap-MS			UHPLC–TOF-MS		
		Recovery (n = 3)[a], %	Intra-day precision (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %
α-HBCD	200	109	3.1	3.5	114	4.2	4.2	104	4.5	5.2
β-HBCD	200	103	3.1	3.8	111	6.6	7.0	102	5.0	5.1
γ-HBCD	200	107	3.4	3.7	112	4.6	4.8	99	2.3	4.3

2nd validation level

Compound	Spiking level, pg g^{-1} f.w.	UHPLC–QqQ-MS/MS			UHPLC–Orbitrap-MS			UHPLC–TOF-MS		
		Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %
α-HBCD	1000	107	2.2	2.8	106	4.1	7.8	110	2.9	3.0
β-HBCD	1000	104	6.1	6.5	105	2.5	5.1	112	2.9	3.5
γ-HBCD	1000	103	2.1	2.5	105	1.6	2.8	106	2.5	2.9

3rd validation level

Compound	Spiking level, pg g^{-1} f.w.	UHPLC–QqQ-MS/MS			UHPLC–Orbitrap-MS			UHPLC–TOF-MS		
		Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %	Recovery (n = 3)[a], %	Intra-day precision, (n = 3)[b], %	Inter-day precision (n = 3)[c], %
α-HBCD	2000	104	1.3	2.7	102	3.0	4.7	105	5.9	5.7
β-HBCD	2000	99	3.7	3.5	103	2.4	3.0	116	7.1	8.1
γ-HBCD	2000	101	2.0	2.0	103	2.7	3.5	101	6.2	6.2

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

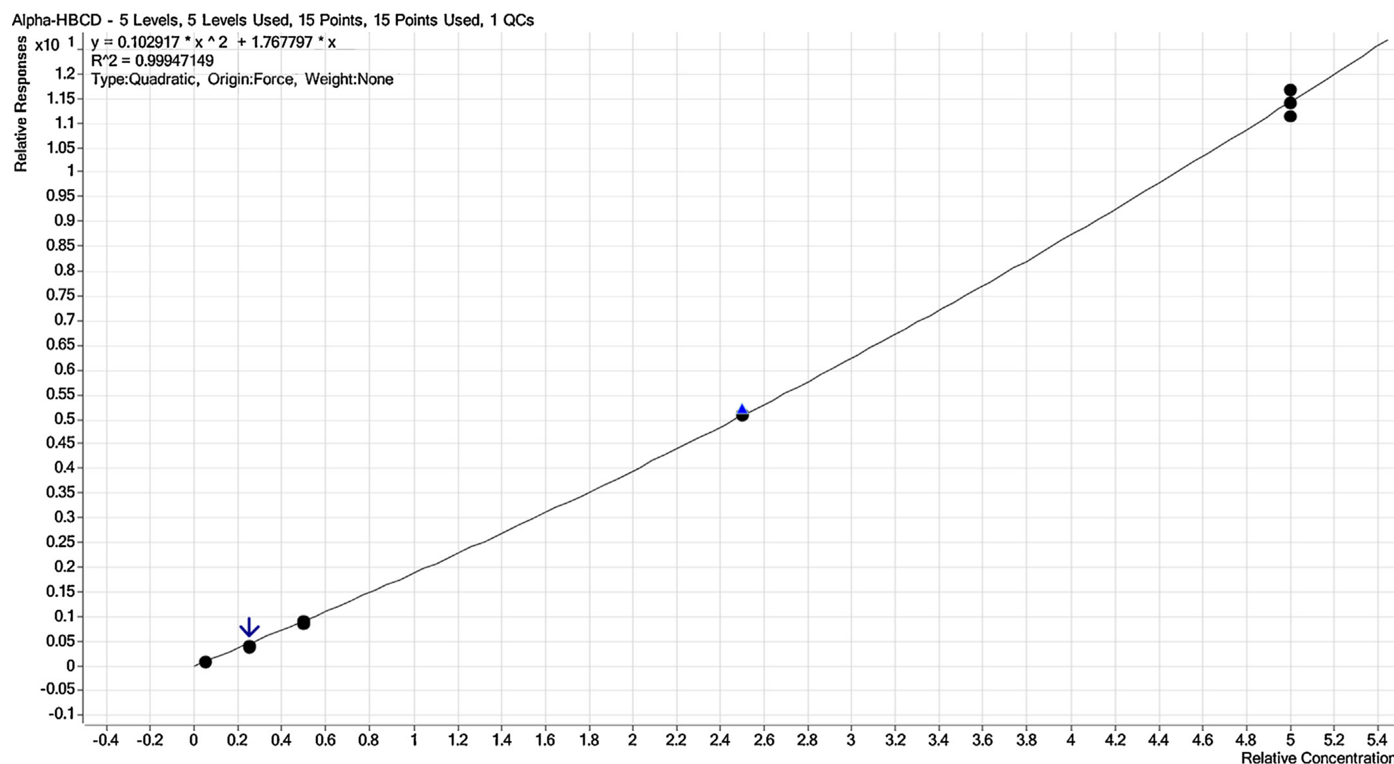

Fig. 3. An example of the matrix matched quadratic type standard curve for α-HBCD within the working range from 1 to 100 pg μL^{-1}, generated by the TOF-HRMS system.

correlation coefficients of 0.995 or greater, and residual values less than 15% for both matrix matched and solvent matched calibration experiments. There were no differences in the plots of calibration curves obtained by matrix matched and solvent matched linearity experiments. Contrary to the Orbitrap-HRMS and QqQ-MS/MS techniques, for TOF-HRMS the non-linear calibration curve was observed within the examined working range from 1 to 100 pg μL^{-1} for all analyzed compounds, thus a quadratic type of the curve was selected as an appropriate fit. There are several mentions in the literature on non-linear behaviour of the TOF-HRMS techniques, which could be attributed to analyte “saturation” effect during the charge competition phenomena inside the analyzer [33–35]. Fig. 3 shows the typical matrix matched quadratic type standard curve for α-HBCD. Although the non-linear relationship for the TOF-HRMS technique may at first glance be considered as a disadvantage for quantitative determination, correlation coefficients of 0.999 or greater were obtained for the quadratic type calibration curves for all three HBCD diastereomers within the working range, and these were successfully used for quantification purposes during the validation experiments and analyses of real samples. Similarly to Orbitrap-HRMS and QqQ-MS/MS techniques, in the case of TOF-HRMS system no difference was observed between the matrix matched and solvent matched calibration plots.

There was a good agreement between the studied LC–MS techniques in terms of recovery, repeatability, and intermediate precision. According to the obtained results, the evaluated instruments demonstrated similar performance in diastereoselective analysis of HBCD. All three applied analytical systems provided adequate recovery values at 200, 1000, and 2000 pg g^{-1} w.w. for each HBCD diastereomer, which were as follows: α-HBCD 102–114%, β-HBCD 99–116%, and γ-HBCD 99–112%. The UHPLC–QqQ-MS/MS system provided a slightly better repeatability and intermediate precision in comparison to the other two studied techniques. The RSD values of run-to-run and day-to-day validation experiments were 1.3–7.1% and 2.0–8.1%, respectively.

The i-LOQ values for the studied HBCD diastereomers were all within a similar range and suitable for confirmatory purposes. The S/N ratio used for the calculation of i-LOQ values was 10:1. The calculated values for i-LOQ for the studied LC–MS techniques were expressed as analyte amount injected on-column and were the following: 1.1–4.5 pg for α-HBCD, 1.4–3.0 for β-HBCD, and 0.7–1.4 pg for γ-HBCD, respectively. The m-LOQ values were assessed by calculations taking into account the sample preparation procedure (sample weight taken for analysis and the final volume of the sample extract), and the signal suppression effect obtained by using acidic treatment procedure with additional purification on Florisil column. The m-LOQs were expressed as pg g^{-1} of sample f.w., and were in the range from 4.0 to 29 pg g^{-1} for α-HBCD, from 7.0 to 13 pg g^{-1} for β-HBCD, and from 4.0 to 7.0 pg g^{-1} for γ-HBCD.

3.5. Performance of the methods in the analysis of Latvian eel samples

In order to compare the applicability of the elaborated LC–MS procedures for analysis of real fish samples, and to obtain the information on the actual levels of HBCD diastereomers in eels (*A. anquilla*) from Latvian lakes, the determination of three HBCD diastereomers in eight samples was performed. The samples were extracted and purified according to the destructive acidic treatment clean-up protocol with the additional purification stage on Florisil column described in Sections 2.3.1, 2.3.2, and 2.3.4. The obtained extracts were analyzed using the above mentioned LC–MS systems as rapidly as possible after the sample preparation to prevent the possible changes of the final extracts. Table 3 summarizes the concentrations of the individual HBCD diastereomers and the total-HBCD values obtained by three different LC–MS techniques. As it could be seen from the results, good agreement was observed between the analytical data obtained by applying the studied LC–MS systems for the analysis of fish samples contaminated with HBCD at sub-ppb levels (Fig. 4). The maximum

Table 3
Concentrations of individual HBCD diastereomers and total-HBCD values for eel (*Anguilla anguilla*) samples, obtained by different LC–MS techniques.

		Sample nr.								Minimum	Maximum	Average[a]
		1	2	3	4	5	6	7	8			
Length, cm		55	50	55	67	55	55	55	50	50	67	55
Weight, kg		0.82	0.98	0.94	1.56	0.87	1.08	0.89	0.85	0.82	1.56	1.00
α-HBCD, pg g^{-1} w.w.	UHPLC–QqQ-MS/MS	144	182	178	157	198	261	356	472	144	472	244
	UHPLC–Orbitrap-HRMS	168	188	168	168	188	269	360	562	168	562	259
	UHPLC–TOF-HRMS	158	170	174	182	217	285	383	511	158	511	260
β-HBCD, pg g^{-1} w.w.	UHPLC–QqQ-MS/MS	44.2	36.0	52.4	32.1	25.9	25.8	18.6	18.8	18.6	52.4	31.7
	UHPLC–Orbitrap-HRMS	60.0	34.8	64.0	35.0	30.0	34.8	<13.0	22.0	<13.0	64.0	36.7
	UHPLC–TOF-HRMS	44.2	33.8	45.5	32.8	29.2	33.8	17.0	31.2	17.0	45.5	33.4
γ-HBCD, pg g^{-1} w.w.	UHPLC–QqQ-MS/MS	17.0	17.2	32.7	19.8	11.4	12.6	<4.00	8.72	<4.00	32.7	15.4
	UHPLC–Orbitrap-HRMS	18.0	17.2	24.0	21.0	13.2	16.0	12.0	12.8	12.0	24.0	16.9
	UHPLC–TOF-HRMS	14.5	15.6	<7.00	13.0	9.34	18.1	<7.00	11.4	<7.00	18.1	12.0
Total-HBCD, pg g^{-1} w.w.	UHPLC–QqQ-MS/MS	206	236	263	209	235	299	380	500	206	500	291
	UHPLC–Orbitrap-HRMS	246	240	256	224	231	320	383	597	224	597	312
	UHPLC–TOF-HRMS	217	219	226	227	255	337	405	554	217	554	305

[a] Average values for HBCD concentrations were calculated as upperbound.

RSD between total-HBCD concentrations obtained for the analyzed samples was 9%. The RSD values between the concentrations obtained for α- and β-HBCDs were in the range of 3–9% and 3–27%, respectively. Higher deviations were obtained at concentrations near the m-LOQ. Thus, for γ-HBCD, which was generally found in the samples at concentrations near the m-LOQ, the RSD values varied from 5% up to 60%. The total-HBCD and individual diastereomer concentrations obtained with each studied LC–MS technique were statistically compared using a Friedman non-parametric statistical test and the probability value (p-value) was evaluated. The p-value was used to decide whether or not the null hypothesis (H_0) according to which the results come from the same population, was true. In the case if the p-value for some of components was smaller than the pre-established significance level ($\alpha = 0.05$), the H_0 was rejected, indicating that the alternative hypothesis could be true. No statistically significant differences were found for α-, β-, γ-, and total-HBCD with the p-values 0.197, 0.054, 0.065, and 0.233, respectively, and therefore these results indicated that the studied techniques produced adequate and similar results on HBCD content in fish samples. However, it should be pointed out that the Friedman test was performed only for samples in which all analytes were detected above the m-LOQ, in order to obtain more realistic results. In the case of calculating p-values for upperbound analyte concentrations (if the analyte was not detected in the sample or detected at concentrations below the m-LOQ, the m-LOQ was used for calculation), γ-HBCD showed p-value of 0.046, thus the H_0 for this component should be rejected. Nevertheless, the factor of statistical significance could not be estimated in such a way with high degree of confidence, because of the inability to provide the real analyte concentration in the sample, but giving only the most pessimistic approximation on the occurrence of the contaminant in the analyzed object. Moreover, the concentrations of β- and γ-HBCDs determined in the analyzed samples were in the range near the m-LOQ (especially for the γ-diasteroisomer), and this could be an additional source of increased dispersion of the results due to the higher measurement uncertainty at these low levels.

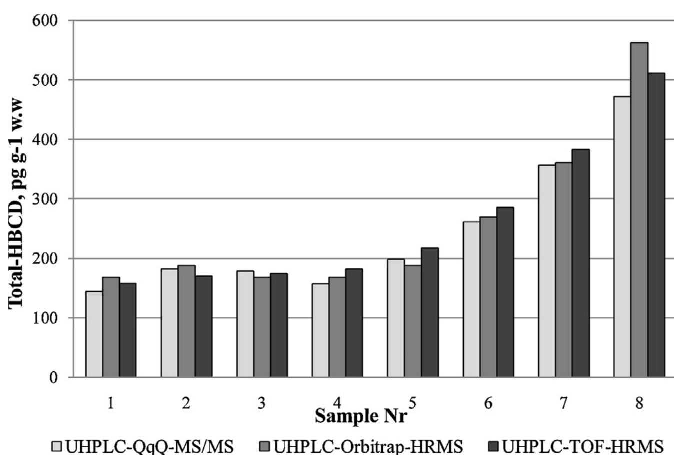

Fig. 4. Comparison of the total-HBCD concentrations in eel samples obtained by applying different LC–MS techniques.

Taking into account the diet of eels ("scavenger" fish) and the typically high fat content, elevated contamination levels of POPs are often encountered in such species [36–39]. Bioaccumulation of HBCD was confirmed in all analyzed eel samples. The total-HBCD concentrations within the samples ranged from 206 to 597 pg g^{-1} w.w, with an average of 303 pg g^{-1} w.w. The biological parameters of the analyzed fishes, concentrations of individual HBCD diastereomers, and the total-HBCD for all analyzed samples are outlined in Table 3. The diastereomer pattern typical for aquatic biota was observed in all samples with the strongly pronounced domination of α-HBCD over β- and γ-HBCDs (up to 95% of the total-HBCD) [40]. Generally, the concentrations determined in this study were significantly lower in comparison to those detected in eel samples analyzed in the majority of earlier studies [37–39]. Some studies indicate strong difference between the HBCD concentrations obtained for eel samples collected from nearby areas. For example, the HBCD concentration in samples collected in the highly polluted south-west region in Netherlands was up to magnitude higher in comparison with the samples collected in relatively remote areas or upstream of the production regions for which total levels of HBCD between 100 and 1000 pg g^{-1} were observed [18]. The relatively low HBCD contamination levels detected in eel samples collected in Latvia seem to be logical, since there are no BFR production factories or plastics processing facilities, which could cause an intensive emission of HBCD into the environment.

4. Conclusions

A rapid and reliable analytical methodology using UHPLC–TOF-HRMS technique has been developed and evaluated to determine three HBCD diastereomers in fish samples. The elaborated method provides good precision over a wide working range and pg g^{-1} quantitation limits. The analytical performance of the UHPLC–TOF-HRMS technique in the analysis of HBCD was comparatively evaluated with two other LC–MS methods: conventional UHPLC–QqQ-MS/MS and modern UHPLC–Orbitrap-HRMS systems operated in SRM and t-SIM, respectively. Comparison of the validation data obtained for three different LC–MS techniques revealed

a good agreement between the studied approaches in terms of recovery, repeatability, and intermediate precision. No statistically significant differences were found for α-, β-, γ-, and total-HBCD concentrations obtained in the analysis of real samples with the examined LC–MS systems, and the results indicate that the studied techniques produced adequate and similar results on HBCD content in fish samples. Overall, the UHPLC–TOF-HRMS operated in scan mode over the m/z range of 600–700 was demonstrated to be a good alternative to conventional LC–MS/MS systems for diastereomer analysis of HBCD and potentially it could be more preferable, taking into account the confirmatory ability and the retrospective post-run evaluation of experimental data for screening of unknown BFRs in the analyzed samples by extension of scanned m/z range and applying the appropriate non-destructive sample preparation procedures. The findings on the contamination status of Latvian eels with HBCD suggest that concentrations of this BFR are lower in comparison to those observed in the majority of published studies, which could be explained with the absence of potential HBCD emission point sources throughout the Latvian territory and nearby region.

Acknowledgments

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