Journal of Chromatography B, 898 (2012) 101–110

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous extraction and determination of HBCD isomers and TBBPA by ASE and LC–MSMS in fish

Guillaume ten Dam[a,b,*], Olga Pardo[c], Wim Traag[a], Martijn van der Lee[a], Ruud Peters[a]

[a] RIKILT – Institute of Food Safety, Akkermaalsbos 2, 6700 AE Wageningen, The Netherlands
[b] Faculty of Science – Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands
[c] Public Health Research Center of Valencia (CSISP), Av. Catalunya, 21, 46020 Valencia, Spain

ARTICLE INFO

Article history:
Received 30 November 2011
Accepted 22 April 2012
Available online 30 April 2012

Keywords:
HBCD
TBBPA
LC–MS/MS
Extraction
Clean-up
Eel

ABSTRACT

Since the EFSA enquired a call for data for TBBPA and HBCD in 2009, the analytical determination of these compounds in food became of regulatory interest. Therefore, a method for the simultaneous determination of TBBPA and the three major HBCD stereoisomers was developed. Conventional techniques like soxhlet, ASE, GPC, sulphuric acid digestion, and acidified silica SPE are generally used in sample pre-treatment while detection is mostly performed by LC–MSMS. A combined analysis of HBCD and TBBPA is problematic due to the hydroxyl groups in the TBBPA molecule. However, using a specific mesh-size sodium sulphate in ASE extraction and an acid silica column combined with a Sep-pack Plus silica cartridge for purification resulted in recoveries between 80% and 110% for all compounds. The accuracy and reproducibility determined using proficiency test samples were 104% and 4% for the sum of the HBCD isomers. Typical limits of detection were 0.01 ng/g product or 0.004 ng on column, while the linear dynamic range is between 0.01 ng and 10 ng on column. Levels of TBBPA and HBCD isomers were determined in eel samples. TBBPA was occasionally detected and only marginally above the quantification limit of 0.05 ng/g, whereas total amounts of HBCD were between 0.2 and 150 ng/g with α-HBCD being the dominant HBCD isomer.

1. Introduction

Hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) (Fig. 1) [1] are commonly used flame retardants in a variety of products. In fact, TBBPA is the most produced brominated flame retardant (BFR) since several years with worldwide production volumes over 100,000 tons per year since 1998 [2,3]. In Europe over 10,000 tons of TBBPA are used annually in products, whereas the annual HBCD usage is approximately 6000 tons [3,4]. The main use of HBCD is in expanded and extruded polystyrene isolation foams and in textile coatings [5]. TBBPA is used in a wide range of electronic devices such as TV sets and PCs and its main application is in printed circuit boards where it is used in more than 95% of the common type of boards. In this application, TBBPA is chemically bound to the epoxy resin and therefore no longer exists as a free chemical in the finally produced board. On the other hand, TBBPA is also used as a non-bound additive BFR in acrylonitrile-butadiene-styrene polymers [6].

In 2008 the European Commission (EC) classified HBCD as a Persistent, Bio accumulative, and Toxic (PBT) compound [7]. With a Log P_{ow} of 5.6, HBCD is likely to absorb to sediments in a water column or to accumulate in fatty tissue [8] and high levels of HBCD have been found in sediments and percolate [9]. Additionally, HBCD has also been found at different levels in fishery products [8–11]. While TBBPA also has a high Log P_{ow} value (4.5–5.3) [12], according to a European risk assessment report it bio-accumulates to a much lesser extent [3]. Nevertheless, TBBPA concentrations up to 400 ng/g have been found in dolphins and concentrations up to 10,000 ng/g have been found in sediments [9]. Unlike HBCD, TBBPA has two acid hydrogens with p*K*a values of 7.5 and 8.5 and the dissociation curves illustrate the susceptibility of the dissociation state of TBBPA towards slight changes around the neutral pH. These p*K*a values should be kept in mind during sample pre-treatment and analysis since the dissociation strongly influences its behaviour during these procedures.

Commercial HBCD is a mixture of mainly α-, β- and γ-HBCD, but δ- and ε-HBCD have also been detected at lower levels [13]. Although the commercial mixture of HBCD contains mostly γ-HBCD, α-HBCD is the predominant isomer in biota due to biotransformation and its water solubility [9,14]. In Europe HBCD production facility can be found in Terneuzen, the Netherlands, while TBBPA is not produced in Europe. As a result it can be

* Corresponding author at: RIKILT – Institute of Food Safety, Akkermaalsbos 2, 6700 AE Wageningen, The Netherlands. Tel.: +31 317480394; fax: +31 317417717.
E-mail address: Guillaume.tendam@wur.nl (G. ten Dam).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.04.025

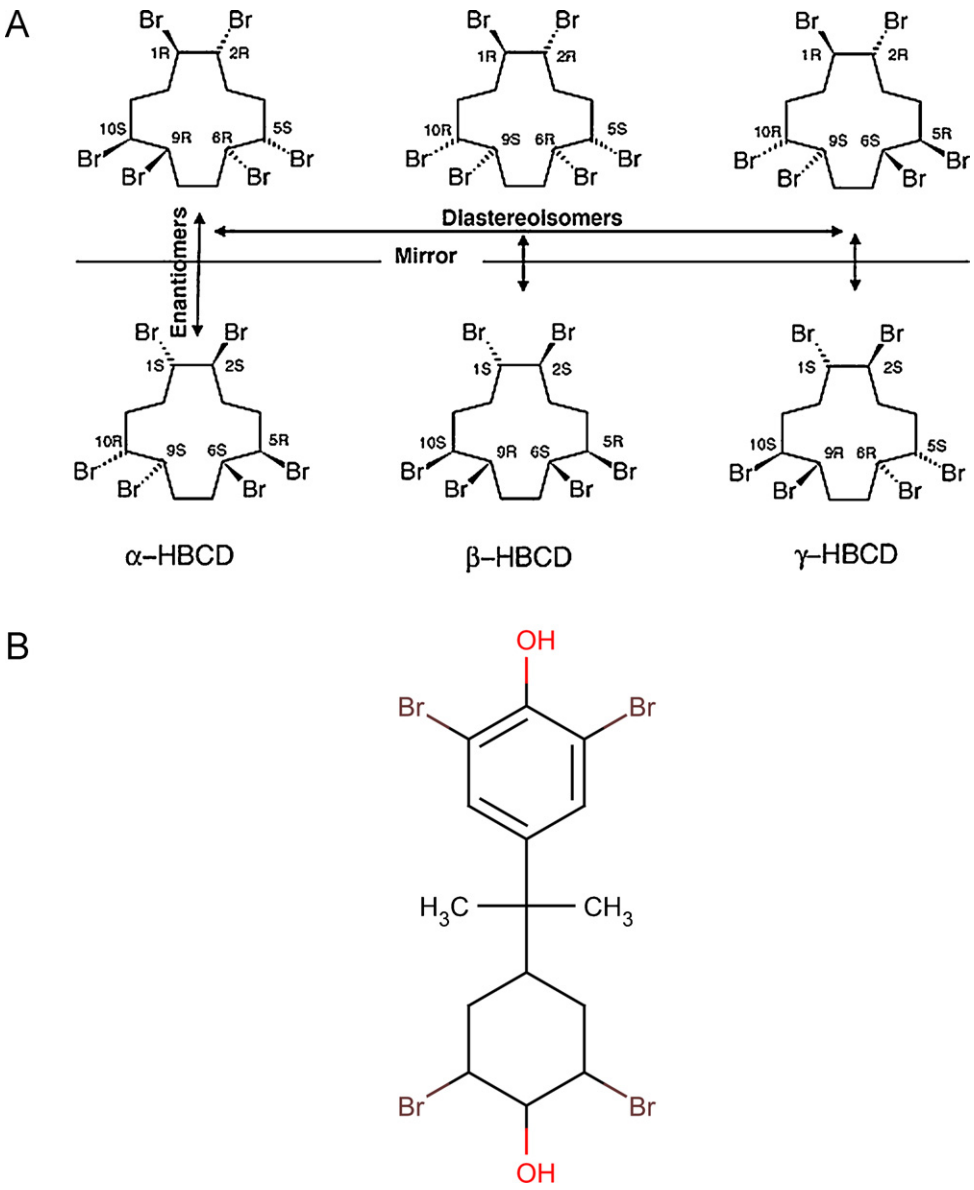

Fig. 1. Structure of the three major HBCD isomers (A) [1] and that of TBBPA (B).

expected that residues of HBCD will be present in the local environment. In previous studies, the presence of HBCD in the Dutch environment has already been demonstrated [11,15]. In general, HBCD concentrations seem to correspond well with overall environmental pollution. Contrary to HBCD, TBBPA is not abundant throughout the environment which could be caused by several reasons. First, TBBPA is often chemically bound to the product [16], secondly, TBBPA is more polar and water soluble than HBCD which might result in lower bio-accumulation [9], and thirdly TBBPA is more easily metabolized and eliminated from organisms [3].

Since both HBCD and TBBPA are widely used in common products and have been found in different parts of the environment, attention has been brought to the governments. As a result, the development of a quantitative analytical method for the determination of the HBCD isomers and TBBPA is not only of scientific interest, but also a regulatory need. Until now several groups describe the analysis of the three major isomers, α, β and γ-HBCD in a variety of environmental and biological samples. However, few are known about their occurrence and combined determinations of HBCD and TBBPA are scarce. Since both compounds are of regulatory interest, a method for the simultaneous determination was developed. In addition, the occurrence of these compounds in eel, which is susceptible to accumulation of hydrophobic compounds, was examined.

Extraction of HBCD is similar to that of conventional contaminants and requires a-polar media, while for TBBPA more polar media are required. Accelarated solvent extraction (ASE) as well as soxhlet extraction have been used to extract HBCD and the extraction conditions are quite consistent throughout the reported studies. For ASE, comparable solvent mixtures are used, but more differences are found regarding temperature, pressure and hydro matrix. The hydro matrix was also sometimes used as clean-up, and florisil [17], acid silica [18] and polyacrylic acid [19] were added to remove interferences or fat from the sample. These alterations do not seem to affect the recovery of the HBCD isomers, but low recoveries for TBBPA has been reported [20,21] (Table 1).

Since HBCD and TBBPA have a high affinity towards apolar media, they disperse into lipid content. Extraction methods are designed to extract the compounds from the matrix, but these methods also extract the lipid content and interfering components.

Table 1
Selected extraction, clean-up and analytical methods.

Reference	Compounds	Matrix	LC column	Eluent	Programme	Extraction	Clean-up
Abdallah [17]	αβγ-HBCD	Dust	Varian Pursuit XRS3 C18 (2 mm × 150 mm, 3 μm)	A MeOH/H_2O 2 mM ammonium acetate, B MeOH	50% A to 0% A to 35% A, 16 min	ASE	H_2SO_4, florisil
Budakowski [22]	αβγ-HBCD	Fish	C18 (2.1 mm x 5000 mm, 4 μm)	A H_2O, B MeOH	30% A to 0% A, 6.5 min	ASE	GPC, florisil
Cariou [44]	αβγ-HBCD, TBBPA	Blood, adipose tissue, milk	Symmetry C18 octadecyl grafted silica (2.1 mm × 150 mm, 3.5 μm)	A MeOH, B ACN, C H_2O 0.5% acetic acid	30:10:60 1 min, 50:50:0 4.5 min	s/l extraction	
Frederiksen [20]	αβγ-HBCD, TBBPA	Biota, egg, liver, adipose tissue	Zorbax C18 (150 mm)			Soxhlet	GPC, H_2SO_4, silica
Granby [19]	αβγ-HBCD, TBBPA	Fish	Gemini C-18 (2 mm × 200 mm × 3 μm)	A MeOH, B H_2O	50% A to 95% A 10 min	ASE, soxhlet	H_2SO_4
Guerra [33]	αβγ-HBCD, TBBPA	Sediment, biota	Symmetry C18 (2.1 mm × 150 mm, 5 μm)	A H_2O/MeOH (1:3), B MeOH	100% A to 10% A 17 min		
Heeb [13]	αβγ-HBCD	HBCD mixture	a-chiral C18-RP, Nucleosil 100-5 (4 mm × 125 mm)	MeOH H_2O (78:22),			
Janak [8]	αβγ-HBCD	Marine	Symmetry C18 (2.1 mm × 150 mm, 5 μm)	A H_2O/MeOH/ACN (6/3/1), B MeOH/ACN (5/5)	A to B in 5 min, hold 6 min	Soxhlet	Acidified silica
van Leeuwen [11]	αβγ-HBCD	Marine	Zorbax XDB-C18 (2.1 mm × 150 mm, 3.5 μm)	A ACN, B 0.01 mM ammonium chloride	70% A 4 min, 90%A in 0.1 min hold 3.9 min	Soxhlet	
Morris [9]	αβγ-HBCD	Sediment, biota	Luna C18 (2 mm × 150 mm, 5 μm)	A H_2O 10 mM ammonium acetate, B ACN 10 mM ammonium acetate	80% A to 13% A in 25 min, to 80% A at 36 min	Ultra Turrax	GPC, H_2SO_4, silica
Morris [24]	αβγ-HBCD	Sediment, biota	Luna C18 (2 mm × 150 mm, 5 μm)	A H_2O 20 mM ammonium acetate, B ACN 20 mM ammonium acetate		Ultra Turrax, s/l extraction	GPC, H_2SO_4, silica
Stapleton [10]	αβγ-HBCD	Sea lion	C30 YMC Caotenoid S-5 (4.6 mm × 250 mm)	A H_2O:MeOH 20:80, B MeOH	A to B in 35 min	ASE	GPC, silica
Suzuki [31]	αβγ-HBCD, TBBPA	Sediment H_2O	Develosil C30-UG-5 (2 mm × 150 mm)	A 5% DCM, B 100% DCM	5% DCM to 100% DCM in 20 min hold 10 min	s/l extraction	
Tomy [23]	αβγ-HBCD	Biota, sediments	Vydac 218MS polymeric rp (2.1 mm × 150 mm)	A ACN:H_2O:MeOH 65:23:12 10 mM ammonium acetate, B ACN	A 5 min 150 μl/min, B 7 min 200 μl/min	ASE	GPC, florisil
Ueno [25]	αβγ-HBCD	Biota	Vydac 218MS polymeric rp (2.1 mm × 150 mm)	A ACN:H_2O:MeOH 65:23:12 10 mM ammonium acetate, B ACN	A 5 min 150 μl/min, B 7 min 200 μl/min	Soxhlet	GPC, silica
Yu [28]	αβγ-HBCD	Air, soil	Zorbax SB-C18 rp (4.6 mm × 250 mm, 5 μm)	A MeOH, B ACNe, C H_2O 10 mM ammonium acetate	80:10:10 to 50:40:10 in 18 min, to 30:70:0 at 23 min hold 7 min	Soxhlet	Acidified silica, alumina
Zhou [29]	αβγ-HBCD	Fish	Restek C18 (2.1 mm × 100 mm, 2.2 μm)	A MeOH:H_2O 85:15, B MeOH	100% A to 0% A in 6 min hold 8 min		Acidified silica, carbon
Riddell [42]	All HBCD isomers	Standards	Restek Ultra II C18 (2.1 mm × 50 mm, 1.9 μm) Waters Acquity BEH C18 (2.1 mm × 50 mm, 1.7 μm)	A H_2O:MeOH 55:45, B MeOH:ACN 70:30	90% A 2.5 min to 65% A in 9.5 min		

In order to remove the lipid content and interferences, several clean-up treatments like GPC [10,20,22–25], sulphuric acid oxidation [8,9,17,18,20,21,24,26–29] and solid phase extraction (SPE) [30] have been developed and are routinely used in contaminant analysis (Table 1).

Since HBCD and TBBPA have a high Log P_{ow}, reconstitution solvents need to be sufficiently a-polar to properly dissolve the compounds. Since γ-HBCD has been found to precipitate in acetonitrile [23,24,31], this solvent is unsuitable for reconstitution of sample extracts.

Chromatographic separation of the three main isomers of HBCD by liquid chromatography (LC) seems to be straight forward and most analyses have been performed on C_{18} columns [8,10,13,20,24,28,31]. Since TBBPA has a p*K*a around neutral pH, the analysis should be performed at pH 4 or lower [32] (Table 1).

Detection of HBCD has mostly been performed with electrospray ionisation tandem mass spectrometry (ESI-MS/MS) [33,34], while ESI with single quadrupole mass spectrometry (ESI-MS) [9,24], atmospheric pressure photo ionization APPI-MS/MS, atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [29], and ESI quadrupole linear ion trap (ESI-QqLIT) [35] have also been used. For the MS/MS determination in the ESI negative mode, the transition mostly measured for HBCD is M−H, *m*/*z* 640.6, to bromine, *m*/*z* 78.8, and for TBBPA this is *m*/*z* 542.6–78.8, *m*/*z* 419.8 and *m*/*z* 447.8.

2. Experimental

2.1. Reagents and materials

Standards of native and ^{13}C labelled HBCD isomers were purchased from Greyhound Chromatography (Birkenhead, UK), while standards of native and ^{13}C ring labelled TBBPA were purchased at Cambridge laboratory (Andover, USA). All solvents were purchased from Biosolve (Valkenswaard, the Netherlands) and were of Pesti-s or dioxin grade, water was of Millipore grade. Acetic acid and diethylamine were purchased from Merck (Whitehouse Station, USA) and Sigma–Aldrich (St. Louis, USA). Hydro matrix, anhydrous sodium sulphate and anhydrous sodium sulphate with a mesh size of 0.63–2.0 mm were purchased from Merck (Whitehouse Station, USA). Diatomaceous earth and Hyflo Super Cel® were purchased from Sigma–Aldrich (St. Louis, USA) and Isolute from Biotage (Uppsala, Sweden) For clean-up of the ASE extracts concentrated sulphuric acid (97%) and silica gel 70–230 mesh were purchased at Merck (Whitehouse Station, USA), while Sep-pack Plus silica cartridges (WAT020520) were purchased from Waters (Elstree, UK).

2.2. Instrumentation and methods

2.2.1. Extraction

A 125 ml extraction cell was filled with two filters and approximately 10 g of hydro matrix spiked with 100 ng native TBBPA and α-,β-, and γ-HBCD. The extraction cell was placed in a Dionex ASE-350 (Sunnyvale, USA) and extracted in 3 cycles at 60 °C or 100 °C with n-hexane/acetone (1/1, v/v) [19,28], n-hexane/acetone (1/1, v/v) with 0.01% acetic acid, or n-hexane/acetone (1/1, v/v) with 0.01% diethyl amine at a pressure of 1500 psi. The heating and static time were set to 5 min, the flush volume to 40% and the purge time to 300 s. The extracts were collected in a 250 ml collection flask after which they were transferred over a funnel containing anhydrous sodium sulphate into a Turbovap tube and evaporated till dryness under a stream of nitrogen at a pressure of roughly 1 bar. The compounds were reconstituted in 0.5 ml of a methanol/water (4/1, v/v) mixture containing 100 ng ^{13}C TBBPA and ^{13}C-α-, ^{13}C-β- and ^{13}C-γ-HBCD and transferred to a vial after which the extract was transferred to a press fit vial.

2.3. Clean-up

2.3.1. GPC

GPC was performed with a Gilson (Middleton, USA) 305 HPLC pump, a Gilson (Middleton, USA) 231–401 autosampler and a Gilson (Middleton, USA) 202 fraction collector, controlled by Unipoint (Winnipeg, Canada) v3.3 software. A 2.5 cm × 60 cm column from Spectrum USA Omnilabo (Phoenix, USA) was purchased and was filled with Biobeads SX3, 200–400 mesh (1522750) purchased from Biorad (Hemel Hempstead, UK). A standard mixture containing 100 ng of TBBPA and α-,β-, and γ-HBCD was dissolved in 15 ml ethyl acetate/cyclohexane (1/1, v/v) and 12.5 ml of this solution was injected in the GPC system. Analytes were eluted using a ethylacetate/cyclohexane (1/1, v/v) [9] mixture at a flow of 10 ml/min. Between elution volumes of 30 and 55 ml five fractions of 5 ml were collected and following the fifth fraction a 20 ml fraction was collected. The collected fractions were evaporated and reconstituted as described before (see Extraction). To determine the recovery of TBBPA and α-,β-, and γ-HBCD in fish samples, 2.5 g of fish oil was spiked with a standard mixture of TBBPA and α-,β-, and γ-HBCD and dissolved in 15 ml ethylacetate/cyclohexane (1/1, v/v). 12.5 ml of the solution was injected on the GPC and the fraction between elution volumes of 35 and 75 ml was collected. The collected fraction was evaporated as described before, after which the amount of residual oil was determined. The residue was reconstituted as described before.

2.3.2. Sulphuric acid

Samples containing 0.5 g of fish oil spiked with 100 ng TBBPA and α-,β-, and γ-HBCD were prepared in a reagent tube. The sample was dissolved in 2 ml hexane after which 1 ml of concentrated sulphuric acid was added [20]. The mixture was shaken gently and left to rest till the phases were separated. The organic layer was transferred to a Turbovap tube, and the sulphuric acid was extracted two times more with 2 ml hexane. The organic phase was evaporated, and reconstituted as described before.

2.3.3. Silica and acid silica SPE

Glass columns were packed with glass wool, 0.5 g anhydrous sodium sulphate and 2 g, 4 g, 8 g or 16 g silica gel respectively. The columns were conditioned with 10 ml hexane after which a 0.5 ml hexane solution containing 100 ng TBBPA and α-,β-, and γ-HBCD was brought on top of the column. After washing with 12 ml, 24 ml, 48 ml or 96 ml hexane, the compounds were eluted with 16 ml, 32 ml, 64 ml or 128 ml hexane/ethylacetate (1/1, v/v) and collected in a Turbovap tube. The collected extract was evaporated and reconstituted as before. The procedure for the cartridges was similar to that for the 2 g silica column [25].

Samples contained 2.5 g fish oil, spiked with 100 ng TBBPA and α-,β-, and γ-HBCD in 10 ml hexane. Acidified silica 33% (w/w) was prepared by adding 50 ml concentrated sulphuric acid to 408 g silica gel in a glass bottle. The mixture was shaken firmly until no more lumps were observed. A part of the acidified silica was diluted 1:1 with silica gel to obtain 16.5% (w/w) acidified silica. The column was from bottom till top filled with 5 g anhydrous sodium sulphate, 10 g silica gel, 10 g acidified silica 33% (w/w), 40 g acidified silica 16.5% (w/w) and 5 g anhydrous sodium sulphate. The column was conditioned with 100 ml hexane and the sample was brought on top of the column. Next the column was washed with 400 ml hexane, after which the compounds were eluted with 500 ml hexane/dichloromethane (1/1, v/v) [8]. The collected extract was evaporated and reconstituted as before.

2.3.4. Automated acid silica SPE

The automated SPE was performed on the Power-Prep™ from FMS (Watertown, USA). Two types of columns were investigated for automated SPE clean-up, the high capacity disposable acidic silica column (HCDS-ACD-STD, Campro Scientific, Berlin, Germany) and the classical disposable neutral silica column (CLDS-NSS-060, Campro Scientific, Berlin, Germany). The high capacity disposable acidic silica column consists of a Teflon column of which the first part is filled with Teflon chips and the rest with 45 g of acid silica. The acid silica contains 44% (w/w) of sulphuric acid and has a prescribed capacity of 4 g of fat. The classical disposable neutral silica column consists of 6 g silica.

The high capacity disposable acidic silica column was conditioned with 100 ml hexane at a flow of 10 ml/min after which a sample containing 0.5 g fish oil spiked with 100 ng TBBPA and α-,β-, and γ-HBCD in 25 ml hexane was applied at a flow rate of 5 ml/min. The Power-Prep™ was programmed to draw 35 ml from the sample, so when the sample was almost completely applied, three more times 5 ml was added and transferred to ensure a quantitative sample application. Subsequently, the column was washed with 150 ml hexane and eluted with 500 ml hexane/dichloromethane (1/1, v/v). The fractions were evaporated and reconstituted as before. This experiment was repeated with addition of the classical disposable neutral silica column after the high capacity disposable acidic silica column. Elution patterns were determined by collecting 50 ml fractions.

2.4. LC–MS/MS analysis

Analyses were performed on an Agilent (Santa Clara, USA) 1200 series HPLC equipped with a Waters (Elstree, UK) Symmetry C_{18}, 150 mm × 2.1 mm, 3.5 μm column [33]. The injection volume was 5 μl and the elution flow rate 0.4 ml/min. To test elution patterns of the HBCD isomers and TBBPA, standards of the individual analytes and analyte mixtures at a concentration of 50 ng/ml in methanol were injected and separated using the following LC gradients: from methanol/water (80/20, v/v) to methanol in 15 min [13]: from acetonitrile/water (80/20, v/v) to acetonitrile in 15 min: from methanol/acetonitrile/water (56/24/20, v/v/v) to methanol/acetonitrile (70/30, v/v) in 15 min [8]: from methanol/acetonitrile/water (56/24/20, v/v/v) with 0.01% acetic acid to methanol/acetonitrile (70/30, v/v) with 0.01% acetic acid in 15 min. Detection was performed using a Waters (Elstree, UK) Quattro Ultima in MS/MS negative mode. The capillary voltage was set at 2.5 kV with a cone voltage of 50 V for HBCD and 130 V for TBBPA. The source temperature was set at 150 °C and the desolvation temperature at 400 °C. The cone gas flow was set at 180 l/h and the desolvation gas flow at 550 l/h. The MS/MS settings were optimized by flow injection analysis. The system was optimized for the highest response of the M−H ion by injecting the individual compounds into an LC flow of 0.4 ml/min, followed by an optimisation to obtain the highest response for the selected transitions. Transitions selected for HBCD are m/z 638.8–79 and m/z 640.8–79 (collision energy 13 eV), and transitions selected for TBBPA are m/z 552.9–417.8 (collision energy 41 eV) and m/z 552.9–447.8 (collision energy 33 eV).

2.5. Sample analysis

Samples eel were collected in two areas in the Netherlands, samples egg, mozzarella cheese and salmon were part of the Folkehelse 2011 proficiency test. The eel samples were skinned and the filet was grounded with liquid nitrogen while the samples egg, mozzarella and salmon were homogenized. From the grounded and homogenized samples 10 g was mixed with 6 g Isolute and spiked with 100 ng ^{13}C TBBPA and ^{13}C HBCD labelled internal standards. The mixture was transferred to a 125 ml extraction cell and extracted in the ASE 350 from Dionex with hexane/acetone (1/1, v/v) at 100 °C and 1500 psi in 3 cycles of 5 min each. The heating and static time was 5 min with a flush volume of 40% and a purge time of 300 s. The extracts were filtered through a funnel containing anhydrous sodium sulphate and transferred to a TurboVap tube. Next, the solvent was evaporated until approximately 0.5 ml. Next, 25 ml of hexane was added, the extract was homogenized and purified using the manual multi-bed acid silica SPE method described in the previous paragraph. The collected fraction from SPE was evaporated till dryness and the residue reconstituted in 0.5 ml methanol/water (4/1, v/v). 10 μl of the final sample extract was injected into the LC–MS/MS system and analysed using a gradient from methanol/acetonitrile/water (56/24/20, v/v/v) to methanol/acetonitrile (70/30, v/v) in 9 min. The MS/MS transitions monitored are m/z 638.8–79 and m/z 640.8–79 for HBCD and m/z 552.9–417.8 and m/z 552.9–447.8 for TBBPA.

2.6. Validation

The method was provisionally validated using salmon samples of Folkehelse proficiency test 2011. Multiple analysis ($n = 7$) was performed to determine the reproducibility and accuracy of the method. LOD's were based on 3 times the signal/noise ratio of the added internal standard. The instrumental linear dynamic range was determined by multiple injections of compound standards in the range of 1–1000 ng/ml. The robustness of the method was determined by the variation of the recovery of the added internal standards.

3. Results and discussion

3.1. Method development

3.1.1. Extraction

The development of a simultaneous extraction method for TBBPA and the three most common HBCD isomers by ASE, started with a study of the influence of the hydro matrix material on the recovery (Fig. 2). The results showed that only one of the five hydro matrices, sodium sulphate with mesh size 0.63–2.0 mm, gave satisfying recoveries for all analytes. Of the analytes, TBBPA was the most sensitive towards the choice of hydro matrix, leading to low and varying recoveries. Although this may have troubled earlier studies [19,20], low recoveries have also been accounted to binding of TBBPA to endogenous matrix compounds in muscle tissue [36]. The fact that all three silica based hydro matrices, diatomaceous earth, hyflo super cell and Isolute result in low and varying recoveries can be explained by the presence of two hydroxyl groups in the structure of TBBPA. These hydroxyl groups can form hydrogen bonds with free silanol or siloxane groups in the silica skeleton of the hydro matrices. These findings are also supported by the extraction recoveries at different pH values and using Isolute as the hydro matrix (Fig. 3). At pH 4 the hydroxyl groups on TBBPA are fully protonated, as a result the hydroxyl groups will undergo maximum hydrogen bonding with the silanol groups resulting in low extraction recoveries, whereas at pH 11 TBBPA is fully dissociated and hydrogen bonding is virtually absent, leading to recoveries of nearly 100%. Particle size is also of importance. Sodium sulphate (mesh size 0.63–2.0 mm) contains much larger particles than diatomaceous earth, hyflo super cell and especially regular anhydrous sodium sulphate. The recoveries of TBBPA and the HBCD isomers using the "large particle" sodium sulphate material is significantly better when compared to the other materials. From this it was concluded that a lower surface area leads to higher extraction recoveries. The extractions performed at 60 °C and 100 °C both

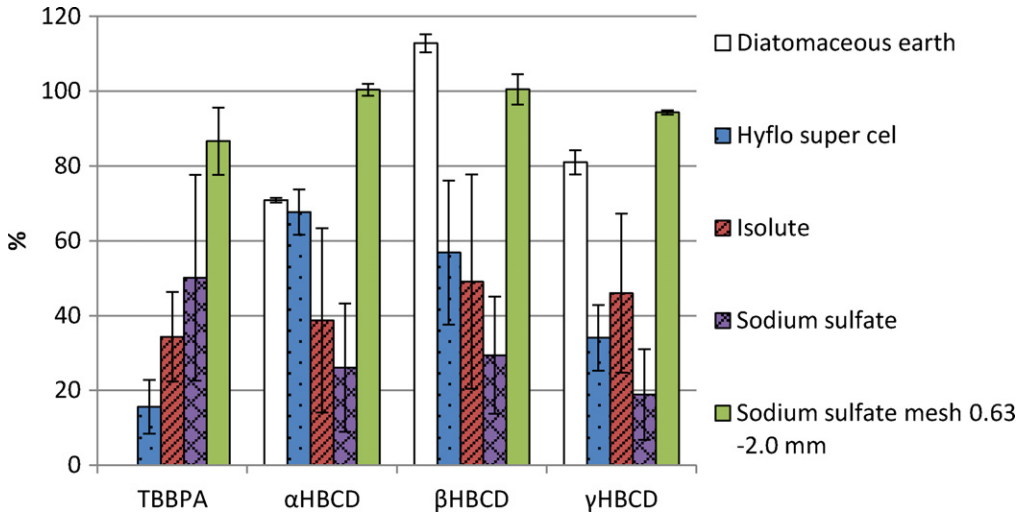

Fig. 2. Recoveries of TBBPA and HBCD isomers in blanks after accelerated solvent extraction (ASE) using different hydro matrix materials. Extraction was performed with a mixture of hexane/acetone (1/1, v/v) at a temperature of 100 °C and a pressure of 1500 psi. The extraction was performed in 3 cycles with a static time of 5 min and a flush volume of 40%. The heating time was 5 min.

resulted in recoveries between 80 and 100% leading to the conclusion that the temperature does not affect the recovery significantly.

3.1.2. Clean-up

3.1.2.1. GPC. The GPC elution patterns TBBPA, HBCD, milk fat and fish oil were determined. The elution window of TBBPA and HBCD ranged from 35 to 50 ml. The majority of the milk fat eluted before 35 ml and approximately 5% of the milk fat eluted within the elution window of TBBPA and HBCD. Overall recoveries after GPC clean-up appeared to be around 100% when summing the amounts of the five individual fractions. However, a compromise between recovery and fat removal will lead to a decrease in recovery.

3.1.2.2. Sulphuric acid. Recoveries of the compounds in the sulphuric acid treatment varied from 40 to 80% for the different compounds with a repeatability of 20%, and taking into account that the recovery of added internal standard in the LC–MS/MS analysis was only 20–40%. These poor recoveries and repeatability's probably originate from insufficient clean-up or co-extracted sulphuric acid. Whatever reason, it is clear that the sulphuric acid clean-up procedure interferes with the LC–MS/MS analysis. Based on the results it cannot be concluded whether compounds are lost during the clean-up or that losses result from a reduced repeatability in the instrumental analysis. By applying additional clean-up steps the interferences due to the sulphuric acid clean-up may be reduced or even removed [9,17,20,24]. Another improvement could be to improve the phase separation by the centrifugation of the extract after sulphuric acid treatment.

3.1.2.3. Silica and acid silica SPE. Methods for silica clean-up derived from literature [10,20,25] resulted in recoveries between 80 and 100% with standard deviations below 10%. The major problem with this procedure is that the capacity for fat removal is insufficient for fatty samples.

The mixed bed column was capable of removing 2.5 g of fat with recoveries around 80% and standard deviations below 5%. Compared to the silica columns, ethyl acetate could not be used for elution since ethyl acetate is rather polar and will elute

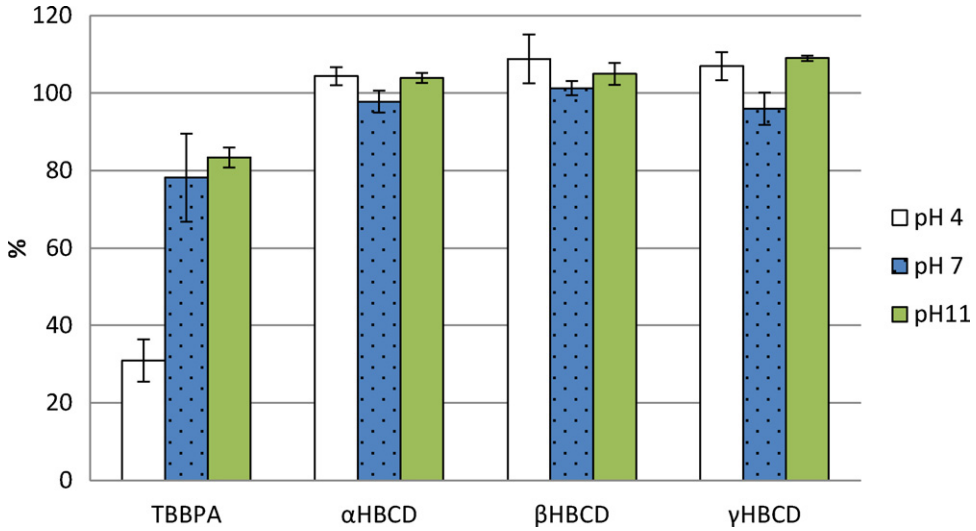

Fig. 3. Recoveries of TBBPA and HBCD isomers in blanks after accelerated solvent extraction (ASE) using Isolute and hexane/acetone (1/1, v/v) containing 0.01% acetic acid (pH 4), hexane/acetone (1/1, v/v) (pH 7) and hexane/acetone (1/1, v/v) containing 0.01% diethylamine (pH 11) at 100 °C and 1500 psi. The extraction was performed in 3 cycles with a static time of 5 min and a flush volume of 40%. The heating time was 5 min.

sulphuric acid from the column. Therefore, dichloromethane was used instead of ethyl acetate. Another problem was clogging of the acidified silica layer of the multi bed column by fat degradation products. For this reason the acid silica layer was replaced by one with a lower sulphuric acid content. In addition, a column with a larger internal diameter was used which resulted in better column permeability.

3.1.2.4. Automated acid silica SPE. Since the manual acid silica SPE required large columns and high solvent volumes, the clean-up procedure was tedious and the sample throughput was relatively low. Therefore, the silica clean-up was automated using an automated SPE system Power-Prep™ from FMS. Since the silica in the high capacity disposable acidic silica column differed from the silica used in the manual (gravitational) silica SPE, the retention of the compounds was different than in the manual approach and the wash and elution volumes had to be adapted to this column [30]. Recoveries of TBBPA and the HBCD isomers between 70 and 100% with a repeatability of 5% were obtained. However, as with the sulphuric acid clean up, matrix interferences were found in the LC–MS/MS analysis, indicating that an additional clean-up of the extracts was required. Additional extract purification using a Sep Pack Plus silica cartridge resulted in good results (Fig. 4). As an alternative to the Sep Pack silica cartridge an additional silica clean-up in line with the high capacity disposable acidic silica column in the FMS system was tested, however, contrary to the previous results, the recovery of TBBPA was only <20%. When determining the elution pattern of the compounds it was found that TBBPA was retained firmly on this column and that during the elution window only small amounts of TBBPA were recovered.

3.1.3. LC–MS/MS analysis

The three major HBCD isomers could be separated using a methanol, acetonitrile and water gradient. It was shown that methanol had a positive effect on the separation of α- and β-HBCD, whereas acetonitrile improved the separation between β- and γ-HBCD. In addition to α-, β-, and γ-HBCD, δ- and ε-HBCD were also injected onto the system and both isomers interfered with another isomer (Fig. 5). While δ-HBCD was still partly separated from α-HBCD, ε-HBCD eluted at the same time as γ-HBCD. If the retention of δ- and ε-HBCD is similar on all C_{18} columns, many results in literature may be overestimated [13]. On the other hand, Dodder et al. found unidentified resolved peaks not being α-, β- or γ-HBCD using an Eclipse C_{18} column [37], while levels of δ- and ε-HBCD in the technical mixture are generally in the order of 0.5%. Since α- and γ-HBCD are present at levels of 10–80% in the technical mixture, quantification of the main isomers might not be affected by the co-elution of other isomers.

When applying the method to samples, retention times of TBBPA shifted depending on the type of sample. Extracts of fish samples were eluting after 2.6 min, while a standard mixture eluted at 3.8 min and a wastewater dilution at 4.6 min (Fig. 6). These differences could be explained by the p*Ka* of TBBPA (pKa_1 7.5) and the pH of the wastewater (pH 5.5). Since TBBPA is almost for 100% protonated at pH 5.5 and therefore will undergo more retention than at neutral pH (standard). The early elution of TBBPA in fish samples is due to a higher pH in the extract (pH > 8). The higher pH leads to dissociation of the hydroxyl groups, making the molecule more polar and thereby minimizing retention. Addition of 0.01% acetic acid to the mobile phase leads to protonation of the hydroxyl groups and results in stable retention times.

MSMS optimized settings were according to the settings in Section 2. MSMS detection of HBCD and TBBPA was straightforward and the transitions reported in the literature could be used. The daughter scan of TBBPA showed three intense fragments, *m*/*z* 79, *m*/*z* 418, and *m*/*z* 448. Since the second transition involves a ring-opening, the same *m*/*z* differences cannot be used to monitor the internal standard ^{13}C TBBPA. Since two transitions originating from the parent ion to different fragments are measured for TBBPA, the analysis of TBBPA meets the criteria for identification points by EU legislation [38]. For HBCD the situation is different since there are only few fragments other than bromine itself. Therefore it was decided to monitor two transitions from the parent ion cluster to bromine. Since both transitions to bromine originate from the same fragment, the analysis of HBCD does not meet the EU criteria.

3.2. Validation

The accuracy of the method for HBCD was 104% with an average concentration of 2.2 ng/g and an assigned value of 2.0 for the sum of the HBCD isomers in Folkehelse proficiency test 2011 [39]. The concentration of the individual isomers was 2.0 ng/g (α-HBCD), 0.03 ng/g (β-HBCD) and 0.11 ng/g (γ-HBCD), while TBBPA was not detected. Since the concentrations of the individual isomers were not reported their accuracies could not be determined, however the concentration of α-HBCD was reported to be 94% [39] of the total amount. The reproducibility standard deviation was determined by analysing the sample material on different days and was found to be 0.078 ng/g (4%) for α-HBCD, 0.0099 ng/g (33%) for β-HBCD and 0.012 ng/g (11%) for γ-HBCD.

Limit of detection (LOD) for HBCD and TBBPA was determined as 3 times the signal/noise ratio of the added internal standard. In biological matrices they were found to be 0.01 ng/g respectively with the exception of egg samples. For egg the recoveries of HBCD were good, however, for TBBPA the recovery was repeatable but only 10% resulting in an LOD of approximately 0.1 ng/g. The linear dynamic range of the relative response factor in the LC/MSMS analyses is from 0.01 ng till 10 ng injected on column. Although the LC column was saturated over 2 ng injected on column, resulting in some distortion of the chromatography, the response remained linear.

3.3. Sample analysis

HBCD was detected in samples salmon and eel (Fig. 7). Since α-HBCD is most prone to bioaccumulation [40] relative high levels of α-HBCD were found compared to β- and γ-HBCD. In known HBCD polluted regions total levels of HBCD up to 134 ng/g were found, whereas in clean areas levels between 0.1 and 1 ng/g were observed. These results are complementary to earlier studies on HBCD, and over the years the potential of HBCD to bio accumulate has been demonstrated, which supports the EFSA commandment [41]. While δ-HBCD and ε-HBCD are present in commercial HBCD mixtures [42], they are not observed in the samples since ε-HBCD co-elutes with γ-HBCD while the relative high concentration of α-HBCD and the limited separation between α- and δ-HBCD make identification of δ-HBCD impossible. HBCD is being produced in the Netherlands in a plant situated in the south-west and the higher HBCD concentrations were found in this region. However, low HBCD concentrations were found in relatively remote areas or upstream of the production area. This indicates that there are also other, probably diffuse sources as emissions from consumer products, of HBCD.

While HBCD was widely found in water samples collected throughout the Netherlands, TBBPA was found only in a few samples of eel at levels up to 1 ng/g. Previous studies also reported low levels and mostly non-detect results for TBBPA. There are a number of reasons that only low TBBPA concentrations are found in aquatic biota. Firstly, TBBPA emissions are probably low since TBBPA is chemically bound in most applications, secondly, TBBPA has a lower bioaccumulation potential compared to other BFR's

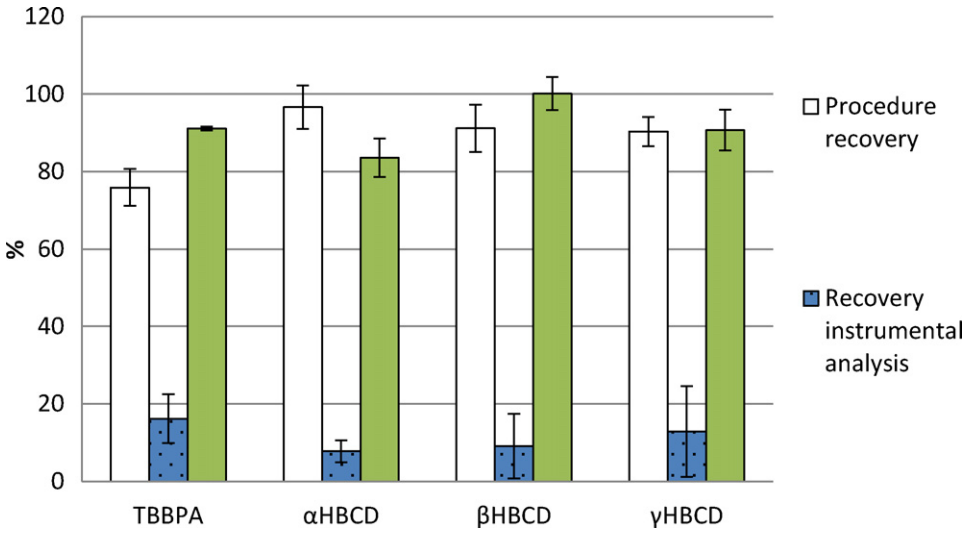

Fig. 4. Accuracy and recovery in sample fish oil after automated SPE. The system was equipped with either a high capacity disposable acidic silica column or a high capacity disposable acidic silica column in line with a Sep-pack plus cartridge. Conditioning was performed with 100 ml hexane at a flow of 10 ml/min, 35 ml of sample solution (0.5 g fish oil in hexane) was applied at a flow rate of 5 ml/min, after which the column was washed with 150 ml hexane and eluted with 500 ml hexane/dichloromethane (1/1, v/v).

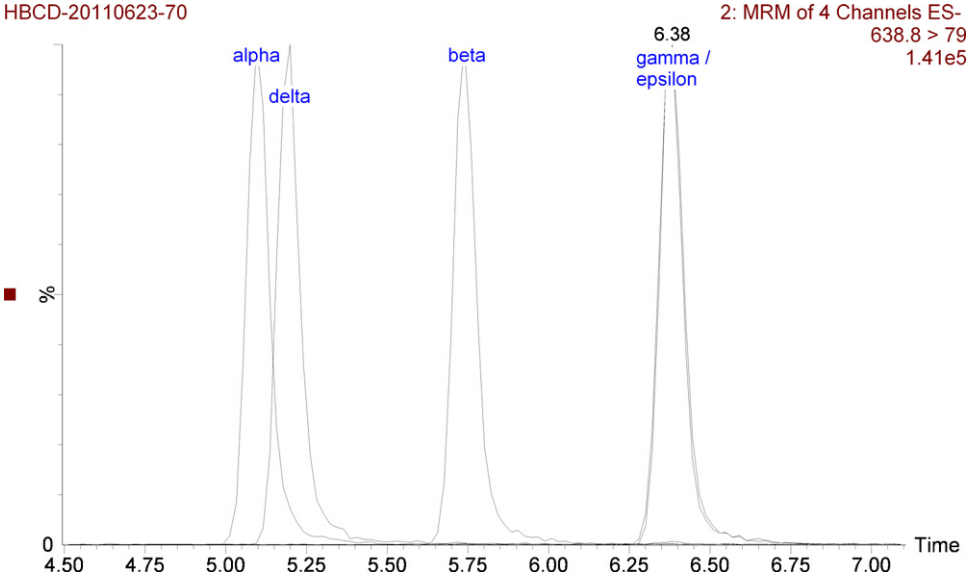

Fig. 5. Chromatograms of 10 μl injections of standard solutions containing 100 ng/ml α-, β-, γ-, δ- and ε-HBCD in methanol/water (4/1, v/v) on a Waters Symmetry C_{18}, 150 mm × 2.1 mm, 3.5 μm column, using a mobile phase A water 0.01% acetic acid, B methanol:acetonitrile 7:3 0.01% acetic acid, and a gradient from 70% B till 95% B in 18 min at a flow of 0.4 ml/min.

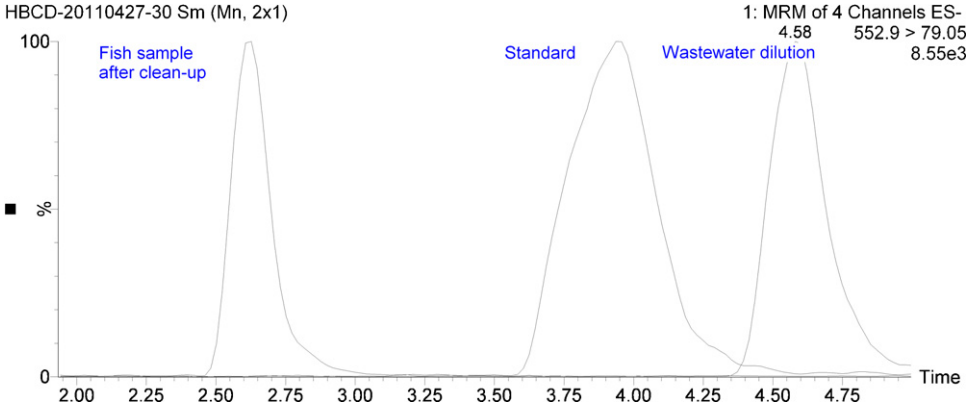

Fig. 6. Chromatograms ^{13}C TBBPA of 10 μl injections of a standard, a fish sample and a waste water sample containing 50 ng/ml ^{13}C TBBPA on a Waters Symmetry C_{18}, 150 mm × 2.1 mm, 3.5 μm column, using a mobile phase of A water, B methanol:acetonitrile 7:3, and a gradient from 80% B till 100% B in 9 min at a flow of 0.4 ml/min.

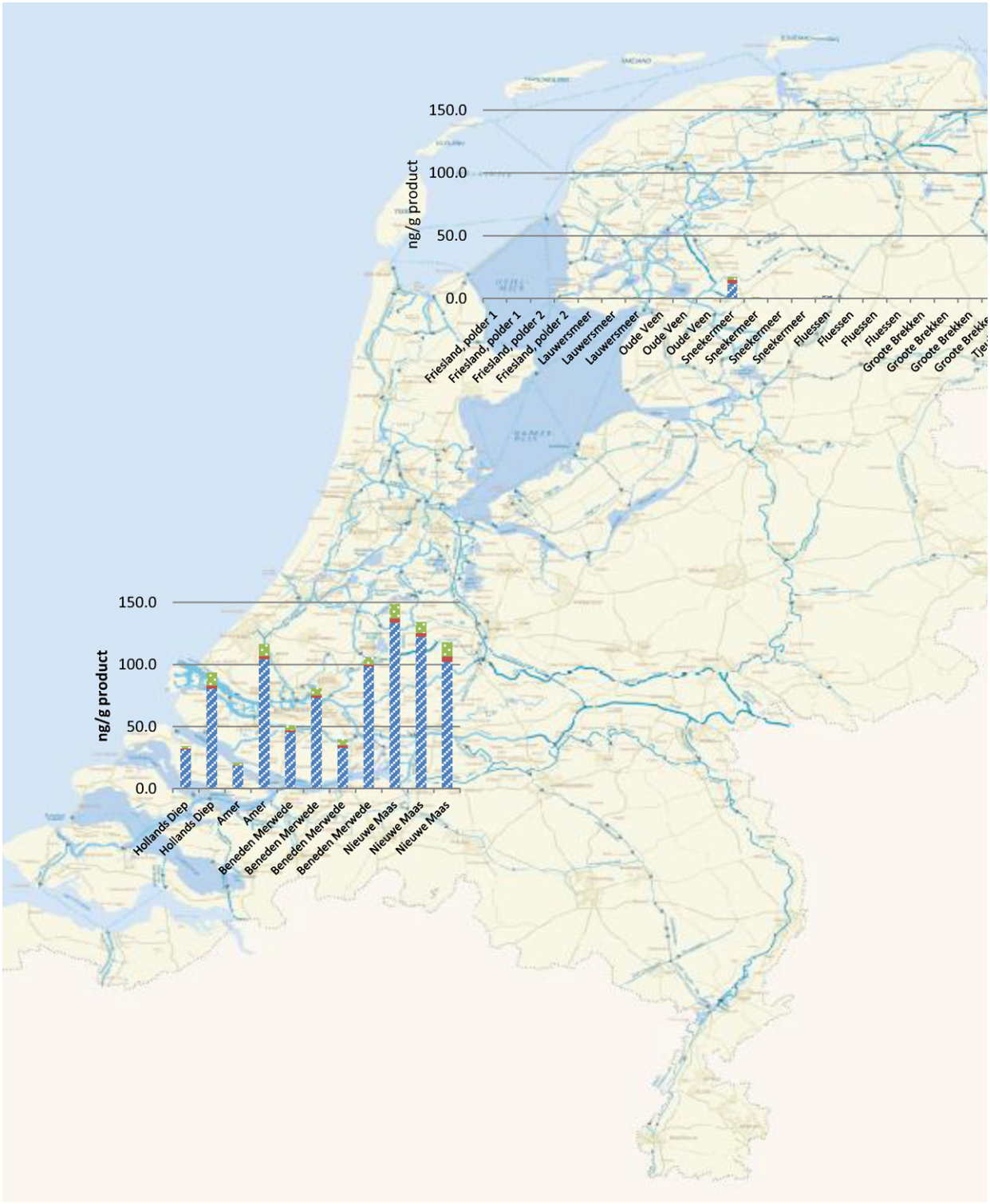

Fig. 7. Concentrations of HBCD in eel in the Netherlands 2009–2010.

as PBDE's, thirdly, the polar nature of TBBPA can subject it to metabolism and elimination from the organisms [14,41,43].

4. Conclusion

An automated method for the combined determination of HBCD isomers and TBBPA in biological samples was developed. The method comprises of ASE extraction followed by automated SPE purification using a Power-Prep™ system, and finally, instrumental analysis with LC/MSMS TBBPA was found to bind easily to silica based materials leading to losses during sample pretreatment. By using non-silica based materials in ASE extraction and a wide elution window in the SPE Power-Prep™ clean-up, TBBPA could be fully recovered from spiked blanks with the exception of egg samples where the recovery of TBBPA was only 10%. Nevertheless, the performance characteristics of the method allow HBCD as well as TBBPA to be measured in biological samples as fish, cheese and eggs at relevant levels. Analysis of samples eel from

the Rhine, Waal and Maas delta, and from lakes in the north of The Netherlands showed that HBCD is present in all samples in concentrations up to 134 ng/g. While α-HBCD is the predominant isomer, β-HBCD and γ-HBCD were also detected in all samples. HBCD was even detected in relatively clean regions of the Netherlands indicating that other, possibly diffuse sources of HBCD are present. TBBPA was found only in a few samples in concentrations up to 1 ng/g.

References

[1] K. Janák, U. Sellström, A.K. Johansson, G. Becher, C.A. de Wit, P. Lindberg, B. Helander, Chemosphere 73 (2008) S193–S200.
[2] BSEF, TBBPA Factsheet, 2004.
[3] European Commission, European Union Risk Assessment Report – TBBP A, 2006.
[4] European Commission, Risk Assessment Report on hexabromocyclododecane, 2008.
[5] BSEF, HBCD Factsheet, 2011.
[6] BSEF, TBBPA Factsheet, 2009.
[7] European Commission, in E.C. Agency (Ed.) ED/67/2008, 2008.
[8] K. Janak, A. Covaci, S. Voorspoels, G. Becher, Environ. Sci. Technol. 39 (2005) 1987.
[9] S. Morris, C.R. Allchin, B.N. Zegers, J.J.H. Haftka, J.P. Boon, C. Belpaire, P.E.G. Leonards, S.P.J. van Leeuwen, J. de Boer, Environ. Sci. Technol. 38 (2004) 5497.
[10] H.M. Stapleton, N.G. Dodder, J.R. Kucklick, C.M. Reddy, M.M. Schantz, P.R. Becker, F. Gulland, B.J. Porter, S.A. Wise, Mar. Pollut. Bull. 52 (2006) 522.
[11] S.P. van Leeuwen, J. de Boer, Mol. Nutr. Food Res. 52 (2008) 194.
[12] WHO, Environmental Health Criteria 172, 1995, Tetrabromobisphenol A and Derivates.
[13] N.V. Heeb, W.B. Schweizer, M. Kohler, A.C. Gerecke, Chemosphere 61 (2005) 65.
[14] S.H. Brandsma, L.T.M. van der Ven, J. de Boer, P.E.G. Leonards, Environ. Sci. Technol. 43 (2009) 6058.
[15] R.J.B. Peters, H. Beeltje, R.J. van Delft, J. Environ. Monitor. 10 (2008) 760.
[16] U. Sellström, B. Jansson, Chemosphere 31 (1995) 3085.
[17] M.A. Abdallah, C. Ibarra, H. Neels, S. Harrad, A. Covaci, J. Chromatogr. A 1190 (2008) 333.
[18] M.H. Berntssen, K. Julshamn, A.K. Lundebye, Chemosphere 78 (2010) 637.
[19] K. Granby, M.K. Nielsen, LC-MS/MS analysis of Hexabromcyclododecane (HBCD) and Tetrabromobisphenol A (TBBPA) flame retardants in food, Danish Institute for Food and Veterinary Research (DFVF), Ministry of Family and Consumer Affairs.
[20] M. Frederiksen, K. Vorkamp, R. Bossi, F. Rigat, M. Dam, B. Svensmark, Int. J. Environ. Anal. Chem. 87 (2007) 1095.
[21] K. Granby, T.L. Cederberg, BFR 2010, 2010.
[22] W. Budakowski, G. Tomy, Rapid Commun. Mass Spectrom. 17 (2003) 1399.
[23] G.T. Tomy, T. Halldorson, R. Danell, K. Law, G. Arsenault, M. Alaee, G. MacInnis, C.H. Marvin, Rapid Commun. Mass Spectrom. 19 (2005) 2819.
[24] S. Morris, P. Bersuder, C.R. Allchin, B. Zegers, J.P. Boon, P.E.G. Leonards, J. de Boer, TrAC Trend Anal. Chem. 25 (2006) 343.
[25] D. Ueno, M. Alaee, C. Marvin, D.C. Muir, G. Macinnis, E. Reiner, P. Crozier, V.I. Furdui, A. Subramanian, G. Fillmann, P.K. Lam, G.J. Zheng, M. Muchtar, H. Razak, M. Prudente, K.H. Chung, S. Tanabe, Environ. Pollut. 144 (2006) 238.
[26] C. Bethune, K. Julshamn, A.-K. Lundebye, Int. J. Food Sci. Technol. 40 (2005) 143.
[27] B. Fangstrom, I. Athanassiadis, T. Odsjo, K. Noren, A. Bergman, Mol. Nutr. Food Res. 52 (2008) 187.
[28] Z. Yu, P. Peng, G. Sheng, J. Fu, J. Chromatogr. A 1190 (2008) 74.
[29] S.N. Zhou, E.J. Reiner, C. Marvin, T. Kolic, N. Riddell, P. Helm, F. Dorman, M. Misselwitz, I.D. Brindle, J. Chromatogr. A 1217 (2010) 633.
[30] E. Mariussen, M. Haukås, H.P.H. Arp, K.-U. Goss, A. Borgen, T.M. Sandanger, J. Chromatogr. A 1217 (2010) 1441.
[31] S. Suzuki, A. Hasegawa, Anal. Sci. 22 (2006) 469.
[32] P. Labadie, K. Tlili, F. Alliot, C. Bourges, A. Desportes, M. Chevreuil, Anal. Bioanal. Chem. 396 (2010) 865.
[33] P. Guerra, A. de la Torre, M.A. Martinez, E. Eljarrat, D. Barcelo, Rapid Commun. Mass Spectrom. 22 (2008) 916.
[34] B. Gomara, R. Lebron-Aguilar, J.E. Quintanilla-Lopez, M.J. Gonzalez, Anal. Chim. Acta 605 (2007) 53.
[35] P. Guerra, E. Eljarrat, D. Barceló, Anal. Bioanal. Chem. 397 (2010) 2817.
[36] B. McHugh, R. Poole, J. Corcoran, P. Anninou, B. Boyle, E. Joyce, M. Barry Foley, E. McGovern, Chemosphere 79 (2010) 305.
[37] N.G. Dodder, A.M. Peck, J.R. Kucklick, L.C. Sander, J. Chromatogr. A 1135 (2006) 36.
[38] European Commision, 2002/657/EC, 2002.
[39] V.L. Horpestad, G. Becher, Interlaboratory Comparison on POPs in Food 2011, Twelfth Round of an International Study, Norwegian Institute of Public Health, Rapport 7 (2011), http://www.fhi.no/dokumenter/3fa30903b1.pdf.
[40] M. Haukås, K. Hylland, T. Nygård, J.A. Berge, E. Mariussen, Sci. Total Environ. 408 (2010) 5910.
[41] EFSA, Request for Data on Brominated Flame Retardants Levels in Foodstuffs, 2009, http://www.efsa.europa.eu/en/dataclosed/call/datex091215.htm.
[42] N. Riddell, R. Becker, B. Chittim, F. Emmerling, R. Köppen, A. Lough, A. McAlees, R. McCrindle, Chemosphere 84 (2011) 900.
[43] EFSA, EFSA J. 9 (2011) 2477.
[44] R. Cariou, J.P. Antignac, P. Marchand, A. Berrebi, D. Zalko, F. Andre, B. Le Bizec, J. Chromatogr. A 1100 (2005) 144.