The MAK-Collection for Occupational Health and Safety

GMS PUBLISSO ZB MED-Publikationsportal Lebenswissenschaften

1,2,5,6,9,10-Hexabromocyclododecane (HBCDD) – Determination of α-HBCDD, β-HBCDD and γ-HBCDD in plasma by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2020

Keywords

1,2,5,6,9,10-Hexabromocyclododecane, HBCDD, flame retardant, biomonitoring, plasma, LC-MS/MS

W. Gries[1]
K. Küpper[1]
C. Schmidtkunz[1]
G. Leng[1]
N. Quinete[2]
A. Alt[2]
T. Schettgen[2]
T. Göen[3,*]
A. Hartwig[4,*]
MAK Commission[5,*]

1 *Method development, Currenta GmbH & Co. OHG, CUR-SER-GS-BLM, Institute of Biomonitoring, 51368 Leverkusen, Germany*

2 *External verification, Institute for Occupational and Social Medicine, University Clinic RWTH Aachen, Pauwelsstraße 30, 52074 Aachen, Germany*

3 *Chair of the working group "Analyses in Biological Materials", Deutsche Forschungsgemeinschaft, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Henkestraße 9–11, 91054 Erlangen, Germany*

4 *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*

5 *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

* *E-Mail: T. Göen (thomas.goeen@fau.de), A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

Citation Note:
Gries W, Küpper K, Schmidtkunz C, Leng G, Quinete N, Alt A, Schettgen T, Göen T, Hartwig A, MAK Commission. 1,2,5,6,9,10-Hexabromocyclododecane (HBCDD) – Determination of α-HBCDD, β-HBCDD and γ-HBCDD in plasma by LC-MS/MS. Biomonitoring Method – Translation of the German version from 2020. MAK Collect Occup Health Saf. 2020 Oct;5(3):Doc068. DOI: 10.34865/bi319455e5_3or

Manuscript completed:
19 Nov 2015

Publication date:
09 Oct 2020

Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

This analytical method allows the selective detection of the flame retardant 1,2,5,6,9,10-hexabromocyclododecane (HCBDD) in plasma, with α-HBCDD, β-HBCDD and γ-HBCDD being the three main stereoisomers of technical HBCDD. The analytes are extracted from plasma after adding the corresponding ^{13}C-labelled internal standards. After precipitation of the plasma proteins and solid phase extraction using a Florisil® cartridge, the analytes are separated from accompanying components by liquid chromatography and analysed using tandem mass spectrometry. Calibration standards are prepared in plasma and processed in the same way as the samples to be analysed.

1 Characteristics of the method

Matrix Plasma

Analytical principle LC-MS/MS

Parameters and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
1,2,5,6,9,10-Hexabromocyclododecane	3194-55-6	1,2,5,6,9,10-Hexabromocyclododecane	3194-55-6
α-HBCDD	134237-50-6	α-HBCDD	134237-50-6
β-HBCDD	134237-51-7	β-HBCDD	134237-51-7
γ-HBCDD	134237-52-8	γ-HBCDD	134237-52-8

Reliability data

α-HBCDD

Within-day precision:	Standard deviation (rel.)	s_w = 21.3%, 13.1% or 10.5%
	Prognostic range	u = 48.2%, 29.6% or 23.8%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg α-HBCDD per litre plasma and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 18.2%, 11.7% or 6.5%
	Prognostic range	u = 41.2%, 26.5% or 14.7%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg α-HBCDD per litre plasma and where n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 95%, 103% or 95%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg α-HBCDD per litre plasma and where n = 10 determinations	
Detection limit:	0.03 µg α-HBCDD per litre plasma	
Quantitation limit:	0.1 µg α-HBCDD per litre plasma	

β-HBCDD

Within-day precision:	Standard deviation (rel.)	s_w = 16.3%, 9.1% or 6.5%
	Prognostic range	u = 37.6%, 20.6% or 14.7%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg β-HBCDD per litre plasma and where n = 9 or 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 14.0%, 10.4% or 10.9%
	Prognostic range	u = 31.7%, 23.5% or 24.7%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg β-HBCDD per litre plasma and where n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 104%, 98% or 97%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg β-HBCDD per litre plasma and where n = 10 determinations	
Detection limit:	0.03 µg β-HBCDD per litre plasma	
Quantitation limit:	0.1 µg β-HBCDD per litre plasma	

γ-HBCDD

Within-day precision:	Standard deviation (rel.)	s_w = 14.0%, 8.7% or 7.2%
	Prognostic range	u = 31.7%, 19.7% or 16.3%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg γ-HBCDD per litre plasma and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 17.6%, 13.5% or 6.9%
	Prognostic range	u = 39.8%, 30.5% or 15.6%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg γ-HBCDD per litre plasma and where n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 95%, 98% or 108%
	at a spiked concentration of 0.1 µg, 1.0 µg or 10.0 µg γ-HBCDD per litre plasma and where n = 10 determinations	
Detection limit:	0.03 µg γ-HBCDD per litre plasma	
Quantitation limit:	0.1 µg γ-HBCDD per litre plasma	

2 General information on HBCDD

1,2,5,6,9,10-Hexabromocyclododecane (HBCDD) is a white odourless powder with a density of 2.24–2.38 g/cm³ and a melting point of 180–185 °C. HBCDD is a highly lipophilic compound with a $\log K_{OW}$ of 5.625 and decomposes at temperatures above 190 °C. Accordingly, its water solubility, which was determined to be approximately 66 µg/l for the technical mixture, is rather poor (NICNAS 2012). It is industrially produced by bromination of

1,5,9-cyclododecatriene. Technical-grade HBCDD mainly consists of the three diastereomers α-HBCDD, β-HBCDD and γ-HBCDD, with each of them existing as pairs of enantiomers. In total, up to 16 stereoisomeric forms are possible (Arsenault et al. 2007 a, b). Depending on the batch, the technical HBCDD mixture consists of approximately 72–90% γ-HBCDD, 9–13% α-HBCDD and < 0.5–12% β-HBCDD (EFSA 2011). The stereoisomers δ-HBCDD and ε-HBCDD, which are also formed during production, are not considered as analytes in the present method due to their low average concentration of < 1% (Heeb et al. 2005, 2008).

Figure 1 shows the chemical structures of α-HBCDD, β-HBCDD and γ-HBCDD.

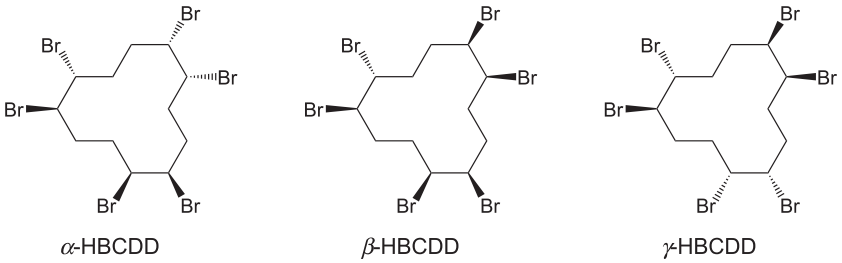

Fig. 1 Structures of the stereoisomers α-HBCDD, β-HBCDD and γ-HBCDD

The production and use of HBCDD is prohibited within the EU since 2016. HBCDD was marketed under various trade names (i.e. Pyroguard®, Pyrovatex®, Saytex®, Myflam®, HBCD or Nicca-Fi-None®) and was primarily used as a flame retardant in expanded or extruded polystyrene. For example, HBCDD was used in polystyrene panels for thermal insulation. It was also used in plastic packaging, in plastic housings for electrical and electronic appliances and in electro-technical components such as cables. Additionally, HBCDD was also used to a lesser extent as textile coating additive and to manufacture upholstered furniture, mattresses or car seats (ECHA 2010 a). Its use as a flame retardant is the result of its ability to reduce flammability and to slow down the fire spread. As an additive, HBCDD is not chemically bound to the polymer matrix but is only incorporated in the plastic matrix (NICNAS 2012).

In 2008, the European Union identified HBCDD as a substance of very high concern (SVHC) and as a persistent, bioaccumulative and toxic substance (PBT substance). Consequently, in 2011 it was listed in Annex XIV of the REACH Regulation (substances subject to authorisation) (ECHA 2010 a; EU 2011 a). In May 2013, HBCDD was included in Annex A of the Stockholm POP Convention on Persistent Organic Pollutants, which was subsequently approved by a respective UN conference (UN 2017). As of 23 March 2016, the production of HBCDD on its own or in preparations has been prohibited (EU 2016). As of 2006, approximately 12 000 tons of HBCDD were used annually in Europe (IOM 2008).

HBCDD may be released from products, to which it was added, by leaching or abrasion during their entire life cycle and can thus be found in air, water or dust (Abdallah and Harrad 2009). This may be due to products from the direct living environment as well as to improperly disposed waste. As HBCDD belongs to the persistent substances it may remain in the environment for a long time. Tests indicate a half-life in soil between 119 and 210 days, with α-HBCDD showing the longest half-life of all HBCDD stereoisomers. Also the elimination of HBCDD from the body appears to be rather slow with an elimination half-life being in the order of weeks to months (ECHA 2010 a). Airborne HBCDD has even been detected in the Arctic region. It is also found worldwide in soil, sediment and water (NICNAS 2012). Due to its high lipophilicity, HBCDD tends to accumulate in the fatty tissue of organisms. The highest concentrations were detected in fish and eggs (EFSA 2011; Janák et al. 2005). Thus, humans may still incorporate HBCDD through various routes of exposure. HBCDD has also been detected in human breast milk, so that infants are as well potentially exposed (Abdallah and Harrad 2011; Eljarrat et al. 2009; Kakimoto et al. 2008; Weiss et al. 2006). Studies on human metabolism are not yet available. However, Geyer et al. (2004) calculated the half-life for humans to be 64 days, using two different approaches.

HBCDD has not been evaluated by the Commission yet. A comprehensive presentation of relevant toxicological studies can be found in the risk assessment report of the EU (2011 b), in the background documents of the European Chemicals Agency (ECHA 2010 a, b) as well as in the document of the Australian National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2012). Reference or limit values in biological material derived by the Commission are not available, but the Human Biomonitoring Commission (HBM Commission) of the German Federal Environment Agency derived a HBM I value of 1.6 µg HBCDD per litre plasma (UBA 2015).

This analytical method allows the selective determination of HBCDD levels in human plasma. To determine possible background exposure, 48 individual plasma samples from individuals not occupationally exposed to HBCDD were analysed. The data obtained with this method are presented in Table 1.

Tab. 1 Background levels of α-, β- and γ-HBCDD in plasma (n = 48)

Analyte	Median [µg/l]	Mean [µg/l]	Number > LOD	Number < LOQ
α-HBCDD	< 0.1	< 0.1	3 (0.03 µg/l, 0.04 µg/l, 0.08 µg/l)	48
β-HBCDD	< 0.1	< 0.1	0	48
γ-HBCDD	< 0.1	< 0.1	0	48

3 General principles

This analytical method enables the selective detection of the flame retardant HCBDD in plasma, with α-HBCDD, β-HBCDD and γ-HBCDD being the three main stereoisomers of technical HBCDD. The analytes are extracted from plasma after adding the corresponding ^{13}C-labelled internal standards. After precipitation of the plasma proteins and solid phase extraction using Florisil® cartridges, the analytes are separated from accompanying components by liquid chromatography and analysed using tandem mass spectrometry. Calibration standards are prepared in plasma and processed in the same way as the samples to be analysed.

4 Equipment, chemicals and solutions

4.1 Equipment

- LC-MS/MS system: Waters Alliance LC 2695 coupled with a Waters Quattro Ultima tandem mass spectrometer (Waters GmbH, Eschborn, Germany)
- LC column: Waters Atlantis T3 3 µm, 3.0 × 50 mm (Waters GmbH, Eschborn, Germany, No. 186003721)
- Laboratory shaker (e.g. IKA-Werke GmbH & Co. KG, Staufen, Germany)
- Pasteur pipettes (e.g. transfer pipettes made of polyethylene, Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Blood collection set with EDTA (e.g. Vacutainer®, Becton Dickinson GmbH, Heidelberg, Germany, No. 367864)
- Various volumetric flasks (e.g. Brand GmbH & Co. KG, Wertheim, Germany)
- 12 ml screw top vials with Teflon-coated screw caps (e.g. SCHOTT AG, Mainz, Germany)
- Variably adjustable pipettes (e.g. Eppendorf AG, Hamburg, Germany)
- Multipette® (e.g. Eppendorf AG, Hamburg, Germany)

- Florisil® SPE cartridges 6 ml/1000 mg (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany, No. 730082.250)
- 200 µl autosampler microvials with crimp caps (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany, No. 702891/702025)
- Refrigerated centrifuge (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be at least p.a. grade.

- Acetonitrile, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 100665)
- Ultrapure water (e.g. Merck KGaA, Darmstadt, Germany, Milli-Q® water)
- Methanol, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 106011)
- Ethanol, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 102371)
- Dichloromethane, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 106054)
- *n*-Hexane, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 104371)
- *iso*-Octane, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 115440)
- Ammonium acetate, Emsure® (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 1.01116)
- α-HBCDD, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. ULM-4834-1.2)
- α-HBCDD-$^{13}C_{12}$, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. CLM-7922-0.5)
- β-HBCDD, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. ULM-5835-1.2)
- β-HBCDD-$^{13}C_{12}$, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. CLM-7923-1.2)
- γ-HBCDD, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. ULM-4836-1.2)
- γ-HBCDD-$^{13}C_{12}$, 50 mg/l in toluene (e.g. Cambridge Isotope Laboratories Inc., Tewksbury, USA, No. CLM-7924-1.2)

4.3 Solutions

- Aqueous ammonium acetate solution (5 mmol/l)

  Exactly 385 mg ammonium acetate are weighed into a 1000 ml volumetric flask and dissolved in ultrapure water. The volumetric flask is then made up to the mark with ultrapure water.

  The solution is stored at room temperature and is stable for at least one week under these conditions.

- Dichloromethane : *n*-hexane 2 : 8 (v : v)

  100 ml dichloromethane are pipetted into a 500 ml volumetric flask. The volumetric flask is then made up to the mark with *n*-hexane.

  The solution is stored at room temperature and is stable for at least six months under these conditions.

4.4 Internal standards (ISTD)

- ISTD spiking solution (1 mg/l)

  200 µl of each stock solution (α-HBCDD-$^{13}C_{12}$, β-HBCDD-$^{13}C_{12}$ and γ-HBCDD-$^{13}C_{12}$) are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with methanol.

  The spiking solution of the internal standards is stored at 4 °C and is stable for at least 12 months under these conditions.

4.5 Calibration standards

- Stock solution (1 mg/l)

  200 µl each of the individual stock solutions (α-HBCDD, β-HBCDD and γ-HBCDD) are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with methanol.

- Spiking solution 1 (0.1 mg/l)

  1 ml of the stock solution is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with methanol.

- Spiking solution 2 (0.01 mg/l)

  100 µl of the stock solution are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with methanol.

- Spiking solution 3 (0.001 mg/L)

  100 µl of spiking solution 1 are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with methanol.

The solutions are stored at 4 °C and are stable for at least 12 months under these conditions.

Calibration standards in a calibration range between 0.05 µg/l and 5 µg/l are prepared according to the pipetting scheme shown in Table 2. Unspiked pooled plasma is included as a blank.

Tab. 2 Pipetting scheme for the preparation of calibration standards used to determine HBCDD in plasma

Calibration standard	Volume of plasma [µl]	Spiking solution	Volume of spiking solution [µl]	Concentration of calibration standard [µg/l]
1	1000	–	–	0
2	950	3	50	0.05
3	990	2	10	0.1
4	980	2	20	0.2
5	950	2	50	0.5
6	990	1	10	1.0
7	980	1	20	2.0
8	950	1	50	5.0

5 Specimen collection and sample preparation

5.1 Specimen collection

After the puncture site has been disinfected, about 5 ml blood are taken from the arm vein of the person to be examined using a blood collection set with anticoagulant additive (EDTA Vacutainer®). The filled tube is then gently inverted to ensure proper mixing of blood and anticoagulant. The blood sample is then centrifuged at 2200 × *g* for ten minutes. The upper plasma phase is transferred into a sealable glass tube using a pipette.

The plasma can be stored in the refrigerator at 4 °C for one week or in the freezer at −20 °C for at least one year.

5.2 Sample preparation

1 ml of the plasma is pipetted into a screw top vial. Using a pipette, 5 µl of the ISTD spiking solution and 200 µl ethanol are added to the sample, which is shaken briefly. 5 ml *n*-hexane are then added to the sample, the vial is sealed and the sample is mixed thoroughly on the laboratory shaker for ten minutes. The sample is then centrifuged at 2200 × *g* for ten minutes at 10 °C.

The SPE cartridge is conditioned using 3 ml dichloromethane and subsequently 8 ml *n*-hexane. Then, the supernatant of the centrifuged sample (hexane phase) is loaded onto the conditioned cartridge using a pipette. The hexane passing through is discarded. Subsequently, the analytes are eluted with 5 ml of the solution of dichloromethane : *n*-hexane 2 : 8 (v/v) into a new vial. The eluate is evaporated to dryness with nitrogen (50 kPa at 35 °C) and the residue is resolved in 100 µl ammonium acetate solution. The sample is then transferred to a 200 µl micro-vial, the vial is sealed and can then be used directly for analysis.

6 Operational parameters

Analysis was performed using a Waters Alliance HPLC system coupled with a Waters Quattro Ultima tandem mass spectrometer.

6.1 High performance liquid chromatography

Analytical column:	Waters Atlantis T3 3 µm, 3.0 × 50 mm
Mobile phase:	A: methanol
	B: aqueous ammonium acetate solution
	C: acetonitrile
Stop time:	15 min
Column temperature:	30 °C
Injection volume:	20 µl
Flow rate:	0.3 ml/min
Sample temperature:	15 °C
Gradient pump:	Gradient program see Table 3

Tab. 3 Gradient pump program

Time [min]	Mobile phase A [Vol.-%]	Mobile phase B [Vol.-%]	Mobile phase C [Vol.-%]
0	20	40	40
1.5	20	20	60
2.5	20	10	70
10	20	10	70
10.05	20	0	80
11	20	0	80
12	20	40	40

All other parameters have to be optimised in accordance with the manufacturer's specifications.

6.2 Tandem mass spectrometry

Ionisation mode:	negative electrospray ionisation (ESI–)
Source temperature:	120 °C
Desolvation temperature:	300 °C
Cone gas flow:	276 l/h
Desolvation gas flow	499 l/h
Collision:	30 eV
Multiplier:	650 V
Collision cell pressure:	9.02×10^{-4} mbar
Collision gas:	argon
Dwell time	0.05 s
Delay	0.1 s
Parameter-specific settings	see Table 4

Tab. 4 Parameter-specific settings for the analytes and the internal standards

Analyte	Retention time [min]	Ion trace [*m/z*]	
		Precursor ion	Product ion
α-HBCDD	7.7	641	79
β-HBCDD	7.9	641	79
γ-HBCDD	8.4	641	79
α-HBCDD-$^{13}C_{12}$	7.7	653	79
β-HBCDD-$^{13}C_{12}$	7.9	653	79
γ-HBCDD-$^{13}C_{12}$	8.4	653	79

The LC-MS/MS product ion spectra of HBCDD-$^{13}C_{12}$ and HBCDD are presented in Figure 2 and the isotope distribution (due to the contained bromine atoms) is shown in Figure 3. The MRM transitions correspond to the fragmenta-

tion of the $[M–H]^-$ pseudomolecular ions to $^{79}Br^-$. The mass 81 (corresponding to $^{81}Br^-$) resulting from the isotope distribution can be used to verify the results. A summation of $^{79}Br^-$ and $^{81}Br^-$ peak areas results in a more sensitive determination of HBCDD.

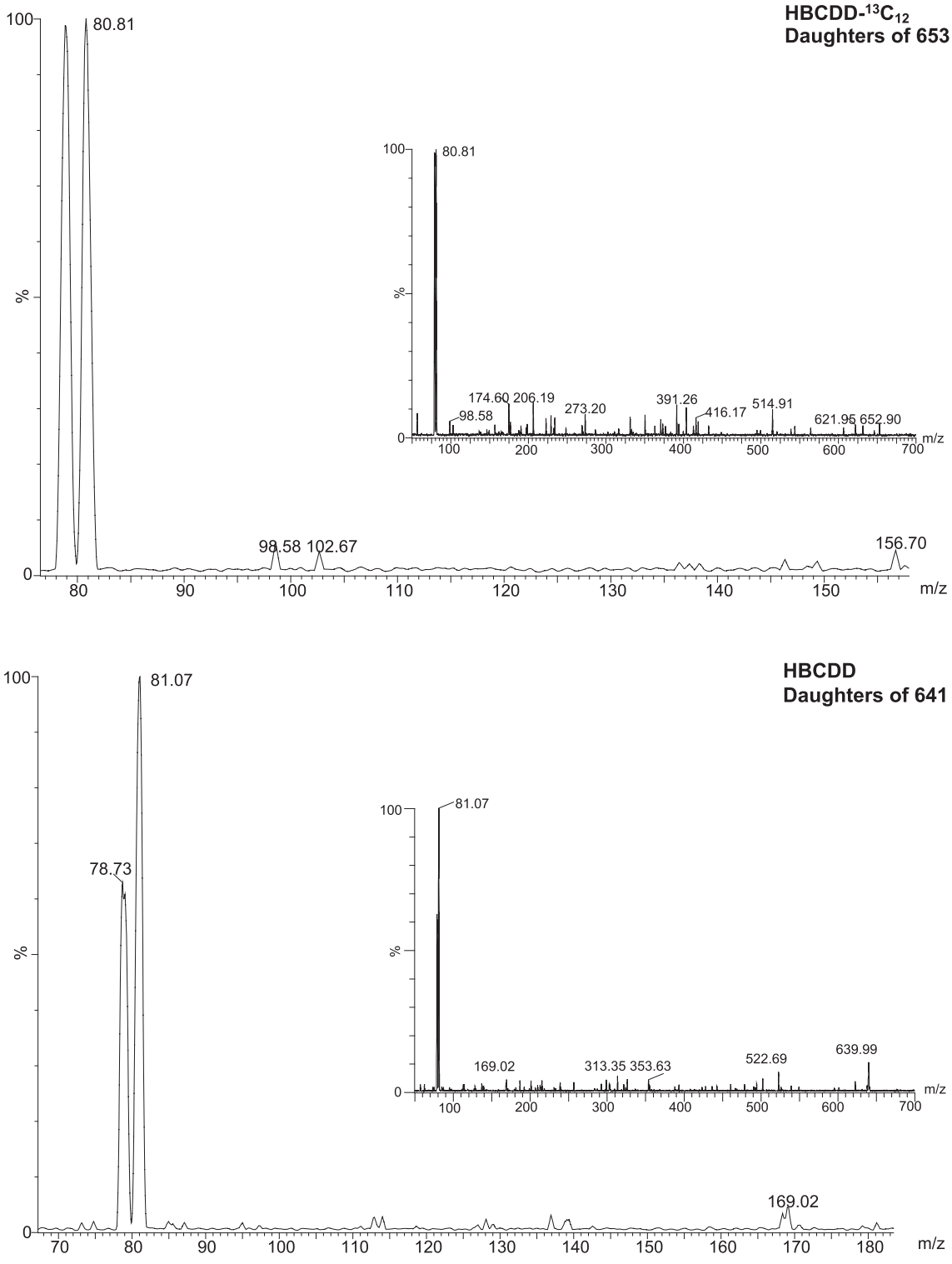

Fig. 2 Product ion spectra of HBCDD-$^{13}C_{12}$ and HBCDD

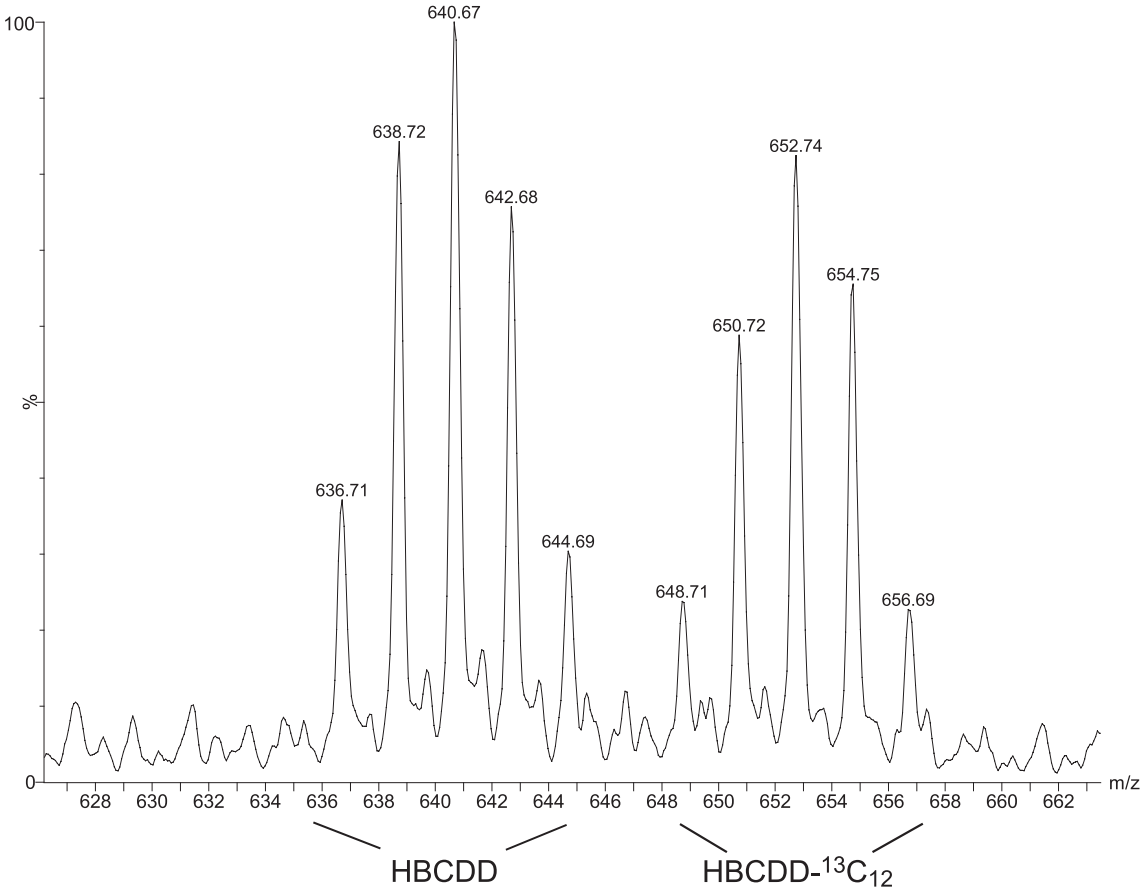

Fig. 3 Isotope distribution of HBCDD-$^{13}C_{12}$ and HBCDD

All settings of the ion source as well as the MRM parameters are instrument-specific and must be adjusted individually by the user. The parameters specified above are therefore intended as a rough guide only.

7 Analytical determination

20 µl of each of the samples prepared as described in Section 5 are injected into the LC-MS/MS system. Identification of the individual analytes is based on the retention times and ion traces of each analyte (Table 4). The retention times given in Table 4 are intended as a rough guide only. Users must ensure proper separation performance of the column used which influences the resulting retention behaviour of the analytes. Figures 4 and 5 show chromatograms of a native plasma sample and of a plasma sample spiked with 0.1 µg/l of each analyte.

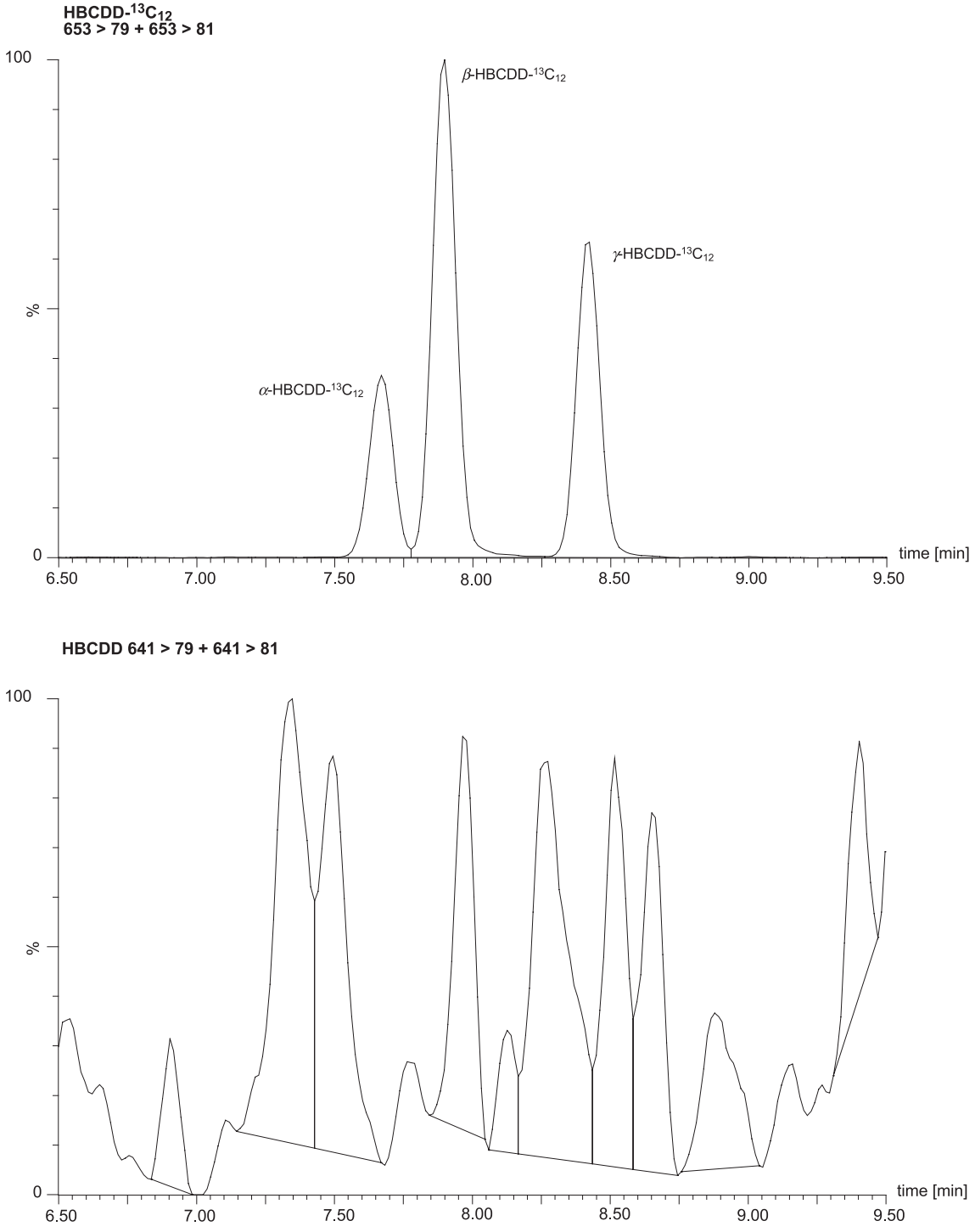

Fig. 4 Chromatogram of a native plasma sample

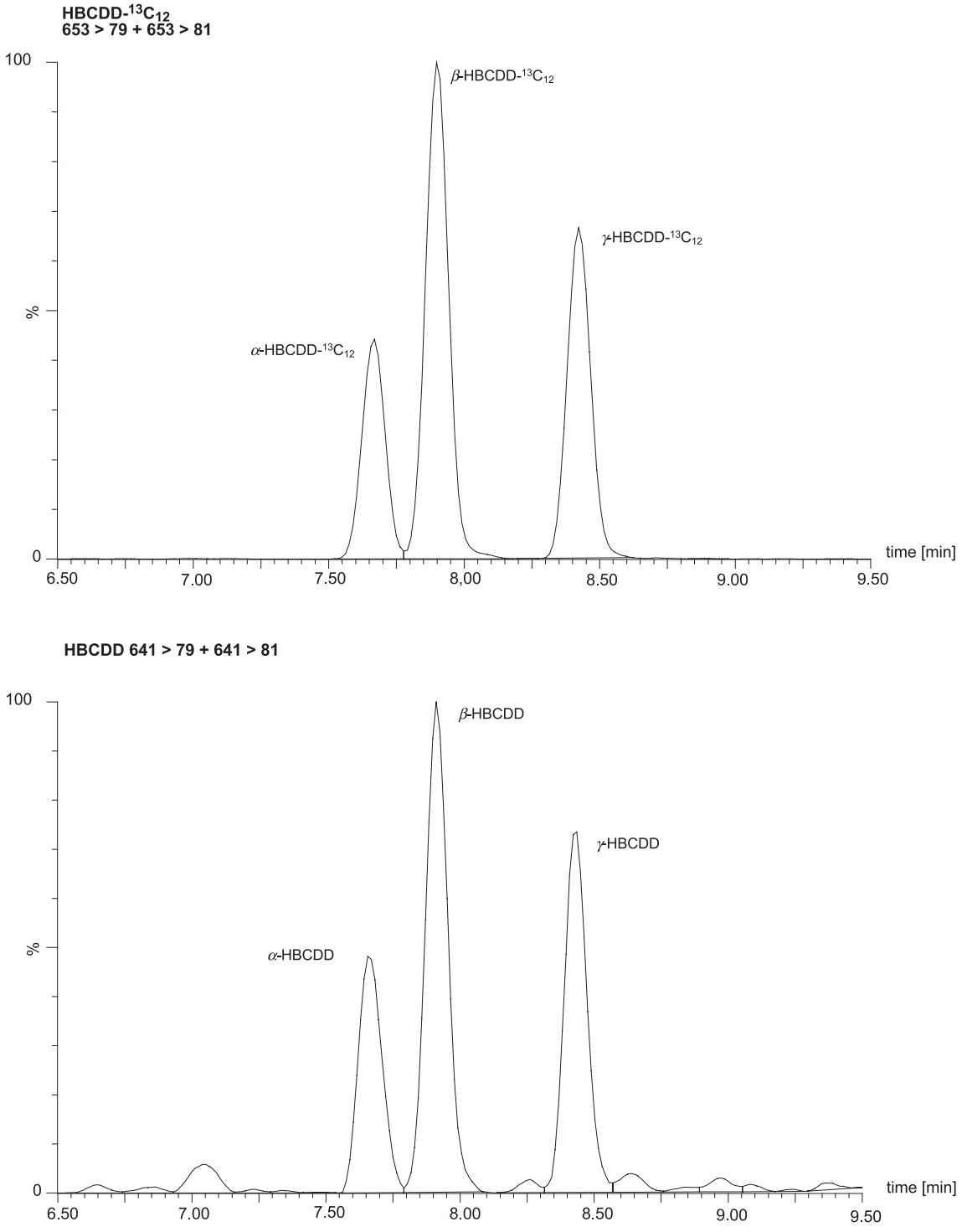

Fig. 5 Chromatogram of a plasma sample spiked with 0.1 µg/l of each of the analytes

8 Calibration

The calibration standards prepared as described in Section 4.5 are processed and analysed according to Section 5.2. Calibration graphs are obtained by plotting the peak area ratios of the respective analyte and of the corresponding ^{13}C-labelled internal standard against the spiked analyte levels. The calibration graph is linear in the concentration range from the detection limit to 100 µg/l. Usually, a calibration range up to 5 µg/l should be sufficient. As an example, Figure 6 shows the calibration graphs for α-HBCDD, β-HBCDD and γ-HBCDD in plasma.

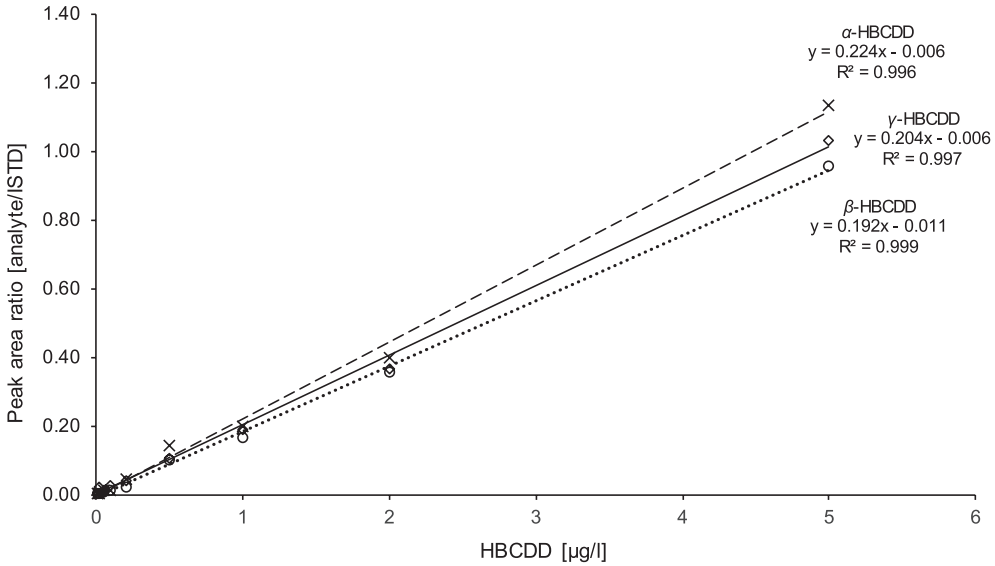

Fig. 6 Calibration graphs for the determination of α-HBCDD, β-HBCDD and γ-HBCDD in plasma

9 Calculation of the analytical results

The analyte concentrations in the plasma samples are calculated using the calibration functions of the respective analytical run (Section 8). The determined peak areas of the individual HBCDD isomers are divided by the peak areas of the corresponding internal standard. The quotients thus obtained are entered into the equation to give the respective analyte concentration in µg/l. Any reagent blank values have to be subtracted from the analytical results.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (*German Medical Association*) and in a general chapter of the MAK-Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014). For quality control, at least three plasma quality control samples with known analyte concentrations are analysed within each analytical run. As control material is not commercially available for the analytes, it must be prepared in the laboratory. To this end, pooled plasma from individuals not occupationally exposed to HBCDD is used and spiked with 0.1 µg/l, 1.0 µg/l or 5 µg/l of each of α-HBCDD, β-HBCDD and γ-HBCDD. Aliquots of these materials are stored at −20 °C and are included in each analytical run as quality control samples. The nominal values and the tolerance ranges of the quality control materials are determined in a pre-analytical period (one analysis of the control materials each on ten different days) (Bader et al. 2010). Additionally, at least one reagent blank is included in each analytical run to identify potential interferences caused by the reagents.

11 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and validation of the method in a second, independent laboratory.

11.1 Precision

Within-day precision was determined using pooled plasma from individuals not occupationally exposed to HBCDD. Ten plasma samples each were spiked with analyte concentrations of 0.1 µg/l, 1.0 µg/l and 10.0 µg/l of each of the analytes. These samples were processed (Section 5.2) and analysed (Section 6) as described. The obtained precision data are presented in Table 5.

Tab. 5 Within-day precision for the determination of α-, β- and γ-HBCDD in plasma (n = 10)

Analyte	Spiked concentration [µg/l]	Determined concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
α-HBCDD	0.1	0.097	21.3	48.2
	1.0	1.03	13.1	29.6
	10.0	10.8	10.5	23.8
β-HBCDD	0.1[a)]	0.099	16.3	37.6
	1.0	1.01	9.1	20.6
	10.0	9.86	6.5	14.7
γ-HBCDD	0.1	0.102	14.0	31.7
	1.0	0.85	8.7	19.7
	10.0	8.96	7.2	16.3

[a)] Outlier eliminated, n = 9

Day-to-day precision was also determined using pooled plasma from individuals not occupationally exposed to HBCDD. Two plasma samples each with analyte concentrations of 0.1 µg/l, 1.0 µg/l and 10.0 µg/l each were processed and analysed on five different days. The precision data thus obtained are presented in Table 6.

Tab. 6 Day-to-day precision for the determination of α-, β- and γ-HBCDD in plasma (n = 10)

Analyte	Spiked concentration [µg/l]	Determined concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
α-HBCDD	0.1	0.095	18.2	41.2
	1.0	1.03	11.7	26.5
	10.0	9.77	6.5	14.7
β-HBCDD	0.1	0.104	14.0	31.7
	1.0	0.98	10.4	23.5
	10.0	9.65	10.9	24.7
γ-HBCDD	0.1	0.095	17.6	39.8
	1.0	0.98	13.5	30.5
	10.0	10.8	6.9	15.6

11.2 Accuracy

The accuracy of the method was determined on the basis of the within-day precision data and the day-to-day precision data. The relative recovery rates thus obtained are presented in Tables 7 and 8.

Tab. 7 Mean relative recovery rates for the determination of α-, β- and γ-HBCDD in plasma, derived from the within-day precision data (n = 10)

Analyte	Spiked concentration [µg/l]	Recovery rate (rel.) *r* [%]	Range [%]
α-HBCDD	0.1	97	68–130
	1.0	103	80–118
	10.0	108	89–120
β-HBCDD	0.1[a)]	99	77–120
	1.0	101	82–113
	10.0	99	87–107
γ-HBCDD	0.1	102	79–125
	1.0	85	76–102
	10.0	90	81–101

[a)] Outlier eliminated, n = 9

Tab. 8 Mean relative recovery rates for the determination of α-, β- and γ-HBCDD in plasma, derived from the day-to-day precision data (n = 10)

Analyte	Spiked concentration [µg/l]	Recovery rate (rel.) *r* [%]	Range [%]
α-HBCDD	0.1	95	74–120
	1.0	103	84–126
	10.0	95	82–118
β-HBCDD	0.1	104	79–120
	1.0	98	85–118
	10.0	97	82–115
γ-HBCDD	0.1	95	65–115
	1.0	98	81–118
	10.0	108	102–115

11.3 Limits of detection and limits of quantitation

The detection limits and quantitation limits were determined on the basis of a signal-to-noise ratio of 3 : 1 for the detection limits and of 9 : 1 for the quantitation limits. The values calculated for the three analytes in plasma are shown in Table 9.

Tab. 9 Limits of detection and limits of quantitation for the determination of α-, β- and γ-HBCDD in plasma (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
α-HBCDD	0.03	0.1
β-HBCDD	0.03	0.1
γ-HBCDD	0.03	0.1

11.4 Sources of error

Various LC columns were tested for their separation performance during method development, but only Nucleodur C18 ISIS (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany, No. 760413.20), XSelect HSS C18 (Waters GmbH, Eschborn, Germany, No. 186006138) and the Atlantis T3 (Waters GmbH, Eschborn, Germany, No. 186003721) were able to achieve stable separation of all three isomers over a longer period of time. The separation of the three HBCDD stereoisomers was not the only selection criterion though. It was observed that a large number of LC columns tested rapidly decreased in separation performance and inertness. This phenomenon was also observed when pure standard solutions were injected, so that the possible accompanying matrix could be ruled out as the cause. For the analyses described herein, two columns, XSelect and Atlantis T3 attained narrow peaks and thus good peak separation of α-HBCDD and β-HBCDD.

The SPE purification technique using Florisil® cartridges is well known from PCB analysis and also proved to be very effective for the determination of HBCDD isomers, as it enabled a selective removal of plasma lipids. A concentration factor of 10 was achieved by evaporating the sample to dryness and taking up the residue in 100 µl of ammonium acetate solution.

The analytical determination by LC-MS/MS showed that the mass fragment *m/z* 79 had a higher intensity compared to *m/z* 81. Quantification via *m/z* 79 and verification via *m/z* 81 resulted in a detection limit of 0.1 µg/l when using a sample volume of 2 ml plasma. Further optimisation steps, such as the increase of the cone gas flow rate and the summation of the peak areas of *m/z* 79 and *m/z* 81, resulted in a distinctly lower detection limit of 0.03 µg/l and a quantitation limit of 0.1 µg/l for each of the three isomers when using a plasma volume of only 1 ml.

The analytical results of this method are expressed in terms of volume (µg/l); however, it should be noted that with regard to HBCDD, there is an international consensus that the analytical results should be expressed in relation to the lipid content of the blood plasma (ng/g lipid). This is based on the phenomenon that highly lipophilic xenobiotics are distributed in body fat and thus the concentration of xenobiotics in blood plasma (or blood serum) depends directly on the lipid levels in this matrix (Phillips et al. 1989). The lipid level, in turn, is dependent on various factors, including food intake in the hours before the blood samples are taken. It can vary within a relatively wide range of 4.5 to 12.6 g lipid/l blood plasma in adults (ICRP 1975).

During external verification, carry-over effects were observed after injection of samples with high analyte levels. To avoid these, it is recommended to inject water after the injection of highly concentrated samples.

12 Discussion of the method

It is a special challenge to quantify HBCDD in biological material. Optimised instrument parameters and an effective sample clean-up are required for the analytical determination of the HBCDD stereoisomers in the given detection range (Covaci et al. 2007; Dodder et al. 2006; Marvin et al. 2007; Thomsen et al. 2008).

This analytical method allows the selective and robust quantification of α-, β- and γ-HBCDD in plasma. The use of only one millilitre of plasma and the quantitation limit of 0.1 µg/l enables the monitoring of occupational exposure (Thomsen et al. 2007) as well as the monitoring of environmental HBCDD exposure, provided that the subjects examined are exposed to specific sources of increased environmental HBCDD exposure (Li et al. 2014; Thomsen et al. 2008). However, the general determination of the background exposure of the general population with this method is not possible (see Table 1). For that purpose, other analytical methods with a higher sensitivity should be used. However, not all of these methods enable a determination of individual HBCDD isomers (Bjermo et al. 2017; Kalantzi et al. 2011; Li et al. 2014; Rawn et al. 2014; Roosens et al. 2009; Tang 2010; Thomsen et al. 2007, 2008; Weiss et al. 2004, 2006).

The use of ^{13}C-labelled internal standards with similar fragmentation patterns is well suited to compensate for various analytical deviations. This was confirmed by the overall very acceptable precision data, as the somewhat

elevated relative standard deviations in the range between 14.0% and 21.3% were entirely due to the recovery tests performed at the quantitation limit of the present method (0.1 µg/l).

In order to obtain an overview of possible background exposures, plasma samples from 48 individuals not occupationally exposed to HBCDD were analysed. No analyte concentrations above the determined limit of quantitation of 0.1 µg/l could be quantified in any of the samples. In three samples, α-HBCDD levels above the LOD were detected, i.e. 0.03 µg/l, 0.04 µg/l and 0.08 µg/l (see Table 1).

During external verification, detection limits of 0.02 µg/l (α-HBCDD and β-HBCDD) and 0.01 µg/l (γ-HBCDD) as well as quantitation limits of 0.05 µg/l (α-HBCDD and β-HBCDD) and 0.03 µg/l (γ-HBCDD) were determined using a different LC-MS/MS system: AB Sciex QTrap 5500 (AB Sciex Germany GmbH, Darmstadt, Germany). Despite the improved quantitation limits, HBCDD could not be quantified in any of 30 plasma samples from the general population. This was further confirmed by a later transfer of the method to a more sensitive LC-MS/MS system (Waters Xevo TQ-S with Waters Acquity UPLC H-Class, column: Acquity UPLC HSS T3, 1.8 µm, 100 Å, 2.1 mm × 100 mm (Waters GmbH, Eschborn, Germany)). In particular, significantly better relative standard deviations for within-day precision (2–9%, instead of 7–21%) and day-to-day precision (2–12%, instead of 7–18%) could be achieved with this instrument, with consistently good recovery rates. The accuracy of this enhanced method has been confirmed by successful participation in interlaboratory tests within the HBM4EU project (Dvorakova et al. 2020; Lankova et al. 2018, 2019 a, b).

Instruments used LC-MS/MS system: Waters Alliance LC 2695 coupled with Waters Quattro Ultima Tandem MS. LC column: Waters Atlantis T3 3 µm, 3.0 × 50 mm. Instrument software: MassLynx 4.1 (Waters GmbH, Eschborn, Germany).

References

Abdallah MA, Harrad S (2009) Personal exposure to HBCDs and its degradation products via ingestion of indoor dust. Environ Int 35: 870–876. DOI: 10.1016/j.envint.2009.03.002

Abdallah MA, Harrad S (2011) Tetrabromobisphenol-A, hexabromocyclododecane and its degradation products in UK human milk: Relationship to external exposure. Environ Int 37: 443–448. DOI: 10.1016/j.envint.2010.11.008

Arsenault G, Chittim B, McAlees A, McCrindle R (2007 a) Nuclear magnetic resonance spectral characterization of confirmation and semi-empirical calculations of confirmations of alpha- and gamma-1,2,5,6,9,10-hexabromocyclododecane. Chemosphere 67: 1684–1694. DOI: 10.1016/j.chemosphere.2006.05.122

Arsenault G, Konstantinov A, Marvin CH, MacInnis G, McAllees A, McCrindle R, Riddell N, Tomy GT, Yeo B (2007 b) Synthesis of the two minor isomers, delta- and epsilon-1,2,5,6,9,10-hexabromocyclododecane, present in commercial hexabromocyclodecane. Chemosphere 68: 887–892. DOI: 10.1016/j.chemosphere.2007.02.005

Bader M, Barr D, Göen T, Schaller KH, Scherer G, Angerer J (2010) Reliability criteria for analytical methods. Biomonitoring Method, 2010. In: Angerer J, Hartwig A (eds) The MAK-Collection for Occupational Health and Safety, Part IV: Biomonitoring Methods, vol 12. Wiley-VCH, Weinheim, 55–101. Also available from DOI: 10.1002/3527600418.bireliabe0012

Bjermo H, Aune M, Cantillana T, Glynn A, Lind PM, Ridefelt P, Darnerud PO (2017) Serum levels of brominated flame retardants (BFRs: PBDE, HBCD) and influence of dietary factors in a population-based study on Swedish adults. Chemosphere 167: 485–491. DOI: 10.1016/j.chemosphere.2016.10.008

Bundesärztekammer (2014) Richtlinie der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen. Dtsch Ärztebl 111: A1583–A1618. https://www.aerzteblatt.de/pdf.asp?id=161921, accessed 08 May 2020

Covaci A, Voorspoels S, Ramos L, Neels H, Blust R (2007) Recent developments in the analysis of brominated flame retardants and brominated natural compounds. J Chromatogr A 1153: 145–171. DOI: 10.1016/j.chroma.2006.11.060

Dodder NG, Peck AM, Kucklick JR, Sander LC (2006) Analysis of hexabromocyclododecane diastereomers and enantiomers by liquid chromatography/tandem mass spectrometry: chromatographic selectivity and ionization matrix effects. J Chromatogr A 1135: 36–42. DOI: 10.1016/j.chroma.2006.09.024

Dvorakova D, Pulkrabova J, Hajslova J (2020) HBM4EU ICI EQUAS report, BFR in serum, round 4. https://www.hbm4eu.eu/mdocs-posts/hbm4eu-ici-equas-report-bfr-in-serum-round-4/, accessed 17 Jun 2020

Eljarrat E, Guerra P, Martínez E, Farré, Alvarez JG, López-Teijón M, Barceló D (2009) Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns. Environ Sci Technol 43: 1940–1946. DOI: 10.1021/es802919e

ECHA (European Chemicals Agency) (2010 a) Committee for Risk Assessment RAC. Annex 1 background document to the opinion proposing harmonised classification and labelling at Community level of hexabromocyclododecane (HBCDD). ECHA/RAC/CLH-O-0000001050-94-03/A1. ECHA, Helsink., https://echa.europa.eu/documents/10162/cd0bb547-00a8-b813-c1df-d54f54e4fdeb, accessed 16 Apr 2020

ECHA (European Chemicals Agency) (2010 b) Annex XV dossier. Proposal for the identification of a substance as a CMR CAT 1 or 2, PBT, vPvB or a substance of an equivalent level of concern. Proposal for identification of hexabromocyclododecane as a SVHC. ECHA, Helsinki. https://echa.europa.eu/documents/10162/3f5de199-8732-4881-aec6-730bf9499a36, accessed 16 Apr 2020

EFSA (European Food Safety Authority) (2011) Scientific opinion on hexabromocyclododecanes (HBCDDs) in food. EFSA J 9: 2296. DOI: 10.2903/j.efsa.2011.2296

EU (European Union) (2011 a) Commission Regulation (EU) No. 143/2011 of 17 Feb 2011 amending Annex XIV to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals ('REACH'). https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02011R0143-20110221&qid=1595335524385&from=EN, accessed 17 Jun 2020

EU (European Union) (2011 b) Risk assessment hexabromocyclododecane. CAS-No. 25637-99-4, EINECS-No. 247-148-4, final report May 2008. https://echa.europa.eu/documents/10162/661bff17-dc0a-4475-9758-40bdd6198f82, accessed 17 Jun 2020

EU (European Union) (2016) Commission Regulation (EU) 2016/293 of 1 Mar 2016 amending Regulation (EC) No. 850/2004 of the European Parliament and of the Council on persistent organic pollutants as regards Annex I. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0293&qid=1594799857275&from=EN, accessed 17 Jun 2020

Geyer HJ, Schramm K-W, Darnerud PO, Aune M, Feicht EA, Fried KW, Henkelmann B, Lenoir D, Schmid P, McDonald TA (2004) Terminal elimination half-lives of the brominated flame retardants TBBPA, HBCD, and lower brominated PBDEs in humans. Organohalogen Compounds 66: 3820–3825. http://dioxin20xx.org/wp-content/uploads/pdfs/2004/04-149.pdf/, accessed 21 Jul 2020

Heeb NV, Schweizer WB, Kohler M, Gerecke AC (2005) Structure elucidation of hexabromocyclododecanes – a class of compounds with a complex stereochemistry. Chemosphere 61: 65–73. DOI: 10.1016/j.chemosphere.2005.03.015

Heeb NV, Schweizer WB, Mattrel P, Haag R, Gerecke AC, Schmid P, Zennegg M, Vonmont H (2008) Regio- and stereoselective isomerization of hexabromocyclododecanes (HBCDs): kinetics and mechanism of gamma- to alpha-HBCD isomerization. Chemosphere 73: 1201–1210. DOI: 10.1016/j.chemosphere.2008.07.045

ICRP (International Commission on Radiological Protection) (1975) Report of the task group on reference man. Pergamon Press, Oxford

IOM (Institute of Occupational Medicine) (2008) Data on manufacture, import, export, uses and releases of HBCD as well as information on potential alternatives to its use. IOM Consulting, supported by BRE, PFA and Entec under framework contract ECHA/2008/2. https://echa.europa.eu/documents/10162/eb5129cf-38e3-4a25-a0f7-b02df8ca4532, accessed 17 Jun 2020

Janák K, Covaci A, Voorspoels S, Becher G (2005) Hexabromocyclododecane in marine species from the Western Scheldt estuary: diastereomer- and enantiomer-specific accumulation. Environ Sci Technol 39: 1987–1994. DOI: 10.1021/es0484909

Kalantzi OI, Geens T, Covaci A, Siskos PA (2011) Distribution of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants in human serum from Greece. Environ Int 37: 349–353. DOI: 10.1016/j.envint.2010.10.005

Kakimoto K, Akutsu K, Konishi Y, Tanaka Y (2008) Time trend of hexabromocyclododecane in the breast milk of Japanese women. Chemosphere 71: 1110–1114. DOI: 10.1016/j.chemosphere.2007.10.035

Lankova D, Pulkrabova J, Hajslova J (2018) HBM4EU ICI EQUAS report, BFR in serum, round 1. https://www.hbm4eu.eu/mdocs-posts/ici-01-bfrs-in-serum/, accessed 17 Jun 2020

Lankova D, Pulkrabova J, Hajslova J (2019 a) HBM4EU ICI EQUAS report, BFR in serum, round 2. https://www.hbm4eu.eu/mdocs-posts/hbm4eu-ici-equas-report-bfr-in-serum-round-2/, accessed 17 Jun 2020

Lankova D, Pulkrabova J, Hajslova J (2019 b) HBM4EU ICI EQUAS report, BFR in serum, round 3. https://www.hbm4eu.eu/mdocs-posts/hbm4eu-ici-equas-report-bfr-in-serum-round-3/, accessed 17 Jun 2020

Li P, Yang CQ, Jin J, Wang Y, Liu WZ, Ding WW (2014) [Correlations between HBCD and thyroid hormone concentrations in human serum from production source area]. Huan Jing Ke Xue 35: 3970–3976. DOI: 10.13227/j.hjkx.2014.10.046, accessed 17 Jun 2020

Marvin CH, MacInnis G, Alaee M, Arsenault G, Tomy GT (2007) Factors influencing enantiomeric fractions of hexabromocyclododecane measured using liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21: 1925–1930. DOI: 10.1002/rcm.3040

NICNAS (National Industrial Chemicals Notification and Assessment Scheme) (2012) Hexabromocyclododecane. Priority existing chemical assessment report No. 34. NICNAS, Sidney. https://www.industrialchemicals.gov.au/sites/default/files/PEC34-Hexabromocyclododecane-HBCD.pdf, accessed 15 Apr 2020

Phillips DL, Pirkle JL, Burse VW, Bernert JT, Henderson LO, Needham LL (1989) Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch Environ Contam Toxicol 18: 495–500. DOI: 10.1007/BF01055015

Rawn DFK, Ryan JJ, Sadler AR, Sun W-F, Weber D, Laffey P, Haines D, Macey K, Van Oostdam J (2014) Brominated flame retardant concentrations in sera from the Canadian Health Measures Survey (CHMS) from 2007 to 2009. Environ Int 63: 26–34. DOI: 10.1016/j.envint.2013.10.012

Roosens L, Abdallah MAE, Harrad S, Neels H, Covaci A (2009) Exposure to hexabromocyclododecanes (HBCDs) via dust ingestion, but not diet, correlates with concentrations in human serum: preliminary results. Environ Health Perspect 117: 1707–1712. DOI: 10.1289/ehp.0900869

Tang C (2010) Quantitative determination of the diastereoisomers of hexabromocyclododecane in human plasma using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 878: 3317–3322. DOI: 10.1016/j.jchromb.2010.10.015

Thomsen C, Molander P, Daae HL, Janák K, Frøshaug M, Liane VH, Thorud S, Becher G, Dybing E (2007) Occupational exposure to hexabromocyclododecane at an industrial plant. Environ Sci Technol 41: 5210–5216. DOI: 10.1021/es0702622

Thomsen C, Knutsen HK, Liane VH, Frøshaug M, Kvalem HE, Haugen M, Meltzer HM, Alexander J, Becher G (2008) Consumption of fish from a contaminated lake strongly affects the concentrations of polybrominated diphenyl ethers and hexabromocyclododecane in serum. Mol Nutr Food Res 52: 228–237. DOI: 10.1002/mnfr.200700123

UBA (Umweltbundesamt) (2015) Stoffmonographie für 1,2,5,6,9,10-Hexabromcyclododecan (HBCDD) – HBM-Werte für HBCDD im Fettanteil der Muttermilch oder des Blutplasmas. Stellungnahme der Kommission „Human-Biomonitoring“ des Umweltbundesamtes. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 58: 889–907. DOI: 10.1007/s00103-015-2193-7

UN (United Nations) (2017) United Nations Environment Programme. Stockholm convention on persistent organic pollutants (POPs). Text and annexes. http://chm.pops.int/Portals/0/download.aspx?d=UNEP-POPS-COP-CONVTEXT-2017.English.pdf, accessed 15 Jun 2020

Weiss J, Meijer L, Sauer P, Linderholm L, Athanassiadis I, Bergman Å (2004) PBDE and HBCDD levels in blood from Dutch mothers and infants - analysis of a Dutch Groningen infant cohort. Organohalogen Compounds 66: 2647–2652. http://dioxin20xx.org/wp-content/uploads/pdfs/2004/04-328.pdf, accessed 21 Jul 2020

Weiss J, Wallin E, Axmon A, Jönsson BA, Åkesson H, Janák K, Hagmar L, Bergman Å (2006) Hydroxy-PCBs, PBDEs and HBCDDs in serum from an elderly population of Swedish fishermen's wives and associations with bone density. Environ Sci Technol 40: 6282–6289. DOI: 10.1021/es0610941