Guidance Document on the Determination of Organobromine Contaminants

ANNEX B

Version 1.0

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Example of Methodology for the Determination of Hexabromocyclododecanes (HBCDDs) in Food and Feed

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Disclaimer

This annex refers to a number of products (analytical standards and equipment) as examples of items that may be used in the described procedures. Alternative sources of these or similar products may be available, and the annex does not endorse or recommend any particular product for use in the described procedures.

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1. Description and Scope

This annex provides details of typical methodologies that may be used for the determination of hexabromocyclododecanes (HBCDDs) in food and animal feed samples. It addresses the analysis of individual HBCDD stereoisomers (alpha-, beta- and gamma-HBCDD) as described in the main guidance document on analytical parameters for the determination of organobromine contaminants. It is based on the use of internal standardisation using labelled surrogates that are commercially available, analyte separation using high or ultra- pressure liquid chromatography (HPLC/UPLC) followed by measurement using high or unit resolution mass spectrometry (LC-Orbitrap, LC-MS/MS). The limit of quantification (LOQ) that is achieved using this method will depend on the sensitivity of the measurement technique used under routine conditions, but laboratories should aim for a LOQ of at least of 0.01 µg/kg wet weight. Although individual HBCDD stereoisomers are targeted which may also be reported as summed concentrations as described in the main guidance document, some of the extraction and purification techniques used may also be used in conjunction with screening of total HBCDD concentrations using GC-MS. In order to allow flexibility of use and incorporation of individual laboratory practices, the methodology for sample extraction, purification and measurement by different LC-MS techniques is presented in modular form (Section 5). Laboratories may choose modules based on available equipment. The description of analytical standards, quantitation and reporting format, described in other sections of this annex is however, common, notwithstanding which modules are chosen.

Module 1: **Description of extraction procedures** (page 6)

- Module 1A: Cold Extraction
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- Module 1C: Hot Pressurized Extraction

Module 2: **Description of purification procedures** (page 9)

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Module 3: **Description of combined manual extraction and manual purification procedure** (page 11)

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- Module 4B: Measurement by LC-OrbitrapMS

Assuming competence in trace analysis and LC-MS, the first steps for a laboratory wishing to set up HBCDD determination are procurement of the required standards and the use of these to establish suitable LC-MS conditions for HBCDD analysis. The described modules are given as examples of working methodology that will allow users to meet the performance requirements given in the main guidance document. As the requirements are performance based, the procedures described here may also be adapted to suit the prevailing expertise/equipment in individual laboratories.

2. Analytical Standards that are used for HBCDD determination

The analytical standards required for the determination of the individual HBCDD stereoisomers listed in the guidance document, are commercially available from current suppliers of persistent organic pollutant (POPs) standards. An example of a full set of standards that could be used for routine determination is listed in Table 1 below.

Table 1: Example of a set of analytical standards (commercially available native, isotopically labelled internal (IS) and recovery standards (RS)) for the determination of HBCDDs

Standard type	HBCDD standard description
Native Standard	1,2,5,6,9,10-hexabromo-(1R,2R,5S,6R,9R,10S)-rel-cyclododecane **(α - HBCDD)**
	1,2,5,6,9,10-hexabromo-(1R,2S,5R,6R,9R,10S)- rel-cyclododecane **(β - HBCDD)**
	1,2,5,6,9,10-hexabromo-(1R,2R,5R,6S,9S,10R)- rel-cyclododecane **(ɣ - HBCDD)**
^{13}C labelled Internal Standard	1,2,5,6,9,10-hexabromo-(1R,2R,5S,6R,9R,10S)-[$^{13}C_{12}$]-rel-cyclododecane **($^{13}C_{12}$ - α-HBCDD)**
	1,2,5,6,9,10-hexabromo-(1R,2S,5R,6R,9R,10S)-[$^{13}C_{12}$]-rel-cyclododecane **($^{13}C_{12}$ - β-HBCDD)**
	1,2,5,6,9,10-hexabromo-(1R,2R,5R,6S,9S,10R)-[$^{13}C_{12}$]-cyclododecane **($^{13}C_{12}$ - ɣ-HBCDD)**
D18 labelled Recovery Standard	d18-1,2,5,6,9,10-hexabromo-(1R,2R,5S,6R,9R,10S)-rel-cyclododecane **(d18 - α-HBCDD)**
	d18-1,2,5,6,9,10-hexabromo-(1R,2S,5R,6R,9R,10S)-rel-cyclododecane **(d18 - β-HBCDD)**
	d18-1,2,5,6,9,10-hexabromo-(1R,2R,5R,6S,9S,10R)-rel-cyclododecane **(d18 - ɣ-HBCDD)**

3. General Advice on Standards and Reagents

It is recommended that reagents of recognized analytical grade and purity (both in terms of HBCDDs and other contamination) are used. It is helpful to check the purity of the reagents and reference materials (e.g. standard solutions) by performing a blank test under the same conditions as used in the method. The resulting chromatograms should be free of the analytes of interest and clear of interferences at the relevant retention times.

- Periodically, check of stability of standards as these may be labile
- Regularly check the volume of standard solutions
- Addition of internal standards (IS) should be before sample pre-treatment and extraction
- Recovery / syringe standards (RS) should be added after the last evaporation step before LC-MS measurement

4. Preparation of Sample material

See advice given in Annex A - PBDE [1] – section 4.

5. Independent Modules on sample extraction, purification and measurement by LC-MS techniques.

5.1 General aspects

The procedures described are directed towards the measurement of individual HBCDD stereoisomers by LC-MS techniques. Screening of the final extracts for total HBCDD by GC-MS may be possible, but in most cases this would require further purification of the extracts.

Depending on the equipment available, either manual or automated procedures may be used. Each module (1, 2, 3 and 4) describes a part of the whole HBCDD determination method. For sample preparation, each extraction method described in Module 1 can be combined with any clean-up method described in Module 2. Module 3 (section 5.4) describes a combined manual extraction and purification method. Each combination of a procedure from Module 1 and 2, and the procedure described in Module 3 can independently be combined with any measurement method describe in Module 4. All modular procedures described here are given as examples and could be adapted or substituted with alternative methods to match the equipment and expertise available in the laboratory.

5.2 Module 1: Extraction

The procedures described in Module 1 are extraction methods for the isolation of HBCDDs from the sample of food or animal feed. The extraction efficiency is affected by the properties of the chosen extraction solvent, the particle size of the raw material, the solvent-to-solid ratio as well as the extraction temperature and the duration/number of cycles of the extraction. The extraction procedures described are divided into cold, hot and hot pressurized extraction and each can be applied to effectively extract HBCDDs from most food or feed sample matrices. Before extraction, the recommendations for preparation of different types of samples should be followed as describe in chapter 4.

For all procedures, representative sample aliquots are internally standardised and extracted using mixed organic solvents or pressurised fluids, either at ambient temperature or higher temperatures depending on the equipment used.

5.2.1 Module 1A: Cold extraction

Principle

Cold solvent extraction is an easy and relatively inexpensive procedure to isolate the analytes of interest from the raw material. The optimum solvent for this purpose penetrates into the solid or wet matrix, the analytes dissolves in the solvent and diffuse out of the matrix. The solvent with lower density separates at the top of the extraction mixture. The supernatant, containing the analytes of interest, can be collected. Multiple different mechanical techniques e.g. shaking, stirring, open column extraction after mixing with drying agent or high-performance dispersion extraction of the fresh sample (according to [2]) are known and may be used if they allow the complete extraction of HBCDD stereoisomers.

Extraction procedure

For high-performance dispersion extraction (using e.g. Ultra Turrax, IKA, Staufen, Germany), isopropanol and cyclohexane are added to the homogenized sample. Immiscible liquid/liquid mixtures or raw powders can be homogenized, dispersed and extracted by high speed stirring. The extraction efficiency is enhanced due to the high shear forces. After addition of water, the sample emulsion is re-extracted. The organic upper phase is collected and the extraction procedure is repeated twice.

5.2.2 Module 1B: Hot extraction

Principle

Continuous hot extraction procedures that operate at temperatures that are close to the boiling point of the used extraction solvent or solvent mixture at atmospheric pressure are an efficient means of extracting HBCDD stereoisomers from the matrix. The high temperature increases solubility and diffusion and therefore enhances the extraction efficiency of the analyte of interest. The extraction is at its most efficient when equilibrium of the solute is reached inside and outside the solid material.

Extraction procedures – manual

Before extraction the sample should be freeze-dried or mixed with a drying agent (e.g. polyacrylate or sodium sulphate). Depending on the composition of the sample, a mixture of cyclohexane and toluene (1/1, v/v) or ethanol and toluene (7/3, v/v) is used, for e.g., Twisselmann extraction (**Figure 1**). The extraction procedure at the boiling point of the solvent mixtures is set to six hours. Permanent hot extraction of the sample takes place with solvent vapour and condensed solvent. A further cleaning of the raw extract with *tert*-butyl methyl ether for fat determination may be necessary

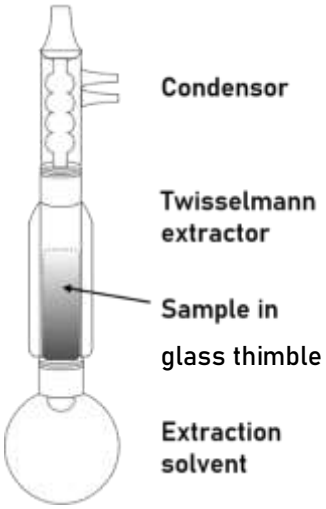

Figure 1: Twisselmann hot extractor with condenser, glass thimble and 500 – 1000 ml round bottom flask

Extraction procedure – automated

A procedure for soxhlet extraction as described in standard EN 16215:2020 [3] for the extraction of PCDD/Fs and PCBs may be used for the extraction of HBCDDs.

The semi-automated Soxtec (Foss, Hilleroed, Denmark) hot extraction for example can be applied for various groups of POPs. Before extraction, the sample aliquot should be freeze dried or mixed with a drying agent. Samples are continuously extracted by an organic solvent mixture heated close to the boiling point at atmospheric pressure. Suitable method conditions can be found in Annex A Table 2 [1] for PBDEs, but may be optimized for HBCDDs to allow appropriate extraction of the stereoisomers.

5.2.3 Module 1C – Hot Pressurized Extraction

Principle

The hot pressurized extraction process uses high temperature and high pressure, which decreases the extraction time and the quantity of solvent used, and shows better repeatability compared to other methods that use less extreme conditions. The high temperature increases solubility and diffusion and therefore enhances the extraction efficiency of the analytes of interest. The high pressure is used to keep the solvent in a liquid state as the temperature is increased above its boiling point, resulting in a higher solubility and higher diffusion rate of lipid solutes into the solvent, and a higher penetration of the solvent into the matrix compared to other less drastic methods.

Extraction procedure – ASE

A procedure for automated pressurized fluid extraction as described in standard EN 16215:2020 [3] for the extraction of PCDD/Fs and PCBs may be used for the extraction of HBCDDs.

Extraction procedure – SpeedExtractor™

Pressurized liquid extraction requires freeze-drying or mixing of the sample with drying agent. The extraction solvents are similar to those used with Soxhlet or Twisselmann hot extraction. Suitable method conditions are summarized in **Table 2.**

Table 2: Examples of ASE and Speedextractor methods applied at EURL

	ASE 200	Speedextractor E-914
Cell volume	**33 ml**	**120 ml**
Weighted sample	10 – 17 g	10 – 30 g
Drying agent	5 – 10 g Hydromatrix 10 g Sodium acrylate/vinyl alcohol copolymer	20 – 50 g Hydromatrix 30 – 50 g Sodium acrylate/vinyl alcohol copolymer
Pressure	10 MPa	10 MPa
Temperature	120 °C	100/120 °C
Static cycles	2	2
Static time	5 min	5 min
N_2 Purge time	1 min	2 min
Extraction cells	33 ml	120 ml

5.3 Module 2 Extract Purification

The procedures described in this module are manual and semi-automated clean-up methods for the isolation of HBCDDs from other components and co-extractives present in a sample of food or animal feed. After extraction of the analytes of interest and evaporation to dryness, the extracted fat fraction is dissolved and an analyte specific purification procedure is carried out. Before the manual clean-up all extraction procedures from section 5.2 can be applied.

Advisory Note: **Reconstitution of final extracts irrespective of purification technique:** Care should be taken during reconstitution if the final extract is partially aqueous because of the low solubility of HBCDDs in water. If possible reconstitution in pure MeOH or ACN is advisable if compatible with the measurement technique that is used.

5.3.1 Module 2A – Manual Clean-up

Principle

Manual or automated purification procedures use broadly similar principles of chromatographic separation of the HBCDDs from residual lipid and other co-extracted interferences, by employing adsorbents such as alumina, silica, Florisil or techniques such as GPC. These internally standardised procedures are carried out using equipment that is commercially available.

Procedure

Accurately weigh 2 g of the extracted fat in an appropriate sample flask, add 2 mL of n-hexane and completely dissolved the fat under ultrasonic treatment and shaking and, if necessary, slight heating (approx. 50 °C).

Using a pipette, fortify the dissolved fat with the required volume of internal standard solution, containing ^{13}C labelled analogues of α-, β- and γ- HBCDD. Typical volumes of a few hundred microliters containing in total, approximately 1 ng of each stereoisomer are suggested, but the standard amount may be varied depending on the level of expected contamination. Allow the added internal standard to infuse and stabilise within the fortified sample.

<u>Purification by silica gel-sulfuric acid column</u>

Preparation and batching of the silica gel-sulfuric acid mixture is based on established PCDD/Fs and PCBs analysis.

Preparation of the column: A large chromatography column (is loaded with a glass wool plug and filled sequentially in the following order:

- 2 g silica gel with 5 % water
- 40 g silica gel/sulfuric acid mixture
- 2 g silica gel with 5 % water

The packed column is then prewashed with 100 ml n-hexane.

Column chromatographic fractionation:

The fat dissolved in 2 ml n-hexane is shaken (e.g., using a Vibrofix device), treated in an ultrasonic bath and applied to the prepared silica gel/sulfuric acid column using a Pasteur pipette. After sample loading, the column is washed with 100 ml of n-hexane while rinsing the sample flask several times. The eluate is discarded. The analytes are then eluted with 150 ml n-hexane/dichloromethane (50+50, v/v) into a 250 ml round bottom flask. Elution should be performed at a drop rate of approximately 1 drop/second. The eluate is concentrated using a rotary evaporator to about 1-2 ml by which time dichloromethane is almost completely removed.

Purification by Florisil column

Preparation of the Forisil column is based on established PCDD/Fs and PCBs analysis.

Preparation of the column: A 30 cm chromatography column is loaded with a glass wool plug and the reservoir filled with n-hexane. 3 g of Florisil is slowly trickled in. Carefully rotation of the column ensures that the Florisil settles evenly. A thin layer of sodium sulfate (1 small spoonful) is added on top of the Florisil and the supernatant n-hexane is drained off.

Column chromatographic fractionation:

The eluate of the silica gel/sulfuric acid column, evaporated to about 1-2 ml, is added to the prepared Florisil column using a Pasteur pipette.

Pre-rinse: 20 ml n-hexane (while rinsing the round bottom flask with the first 5 ml in 2 portions).

Elution: 20 ml n-hexane/dichloromethane (50+50, v/v), collect in a 25 ml round bottom flask with appendix. Elution should be performed at a drop rate of approximately 1 drop/second.

Preparation of the LC/MS measuring solution

The hexane/dichloromethane eluate is now concentrated to approx. 100 µl using a rotary evaporator. Subsequently the solvent residues are gently evaporated to dryness under a nitrogen stream. The residue is dissolved in 100 µl acetonitrile under ultrasonic treatment and shaking.

5.3.2 Module 2B– Semi-Automated Purification

This module describes procedures for the semi-automated clean-up of HBCDDs from other components and co-extractives present in a sample of food or animal feed. This internally standardised procedure is carried out using equipment that is commercially available.

Principle

The extracts derived from either the manual or automated techniques described under section 5.2 above require purification before they can be analysed and quantified. Separation of HBCDDs from other components and co-extractives present in the sample of food or feed can be fully automated by gel permeation chromatography GPC. In particular, GPC would be suitable for the separation of waxes. An additional manual clean-up step and separation from

other analytes applying silica gel column chromatography might still be needed. The semi-automated clean-up is based on established PCDD/Fs and PCBs analysis.

Procedure

0.5 g of the extracted fat is dissolved in 5 ml of a cyclohexane/ethylacetate mixture. GPC is carried out using e.g., Bio Beads S-X3 as the filling material. Exact filling volumes, column size, flow velocity and the elution program depends on the applied instrument. An example is given in Annex A under 5.3.1 [1] of the guidance document for PBDEs.

For the second clean-up step the evaporated GPC extract is purified using a small silicagel column (1g with 1.5% water). HBCDDs are eluted with 10 ml toluene and the eluate is evaporated to dryness.

Preparation of the LC/MS measuring solution

The residue is dissolved in 100 µl acetonitrile or methanol under ultrasonic treatment and shaking.

5.4 Module 3– Combined manual Extraction and manual Purification

The procedure described in this module is a manual method for the isolation and purification of HBCDDs from other contaminants and co-extracted nutrients present in samples of food or animal feed. This internally standardised procedure combines extraction and preliminary purification in a single stage.

Principle

Aliquots of adequate mass (to allow achievement of the LOQ) of the homogenised test material are fortified with the internal standard, stabilised, and dispersed in solution. The solution is passed through an acid modified silica layer where the HBCDDs are isolated from the matrix using hydrolysis of the matrix and cold solvent extraction. (If screening for total HBCDD by GC-MS, further purification of the eluate using an alumina column is required). The eluate is concentrated and solvent exchanged to a methanol:water medium with the introduction of the internal sensitivity standard.

Procedure

Accurately weigh a sub-sample of the homogenised sample material into a sturdy 500 ml glass bottle. The weight will depend on the sensitivity on the measurement process, but for most samples, typically 3 - 10g of material (or 2-3 g lipid equivalent) should be used to ensure a representative sample size and achieve the required LOQ.

Using a pipette, fortify the weighed sample with the required volume of internal standard solution, containing ^{13}C labelled analogues of α-, β- and γ- HBCDD by spiking small droplets of the solution over the cross-section of the sample surface. Typical volumes of a few hundred microliters containing in total, approximately 1 ng of each stereoisomer are suggested, but the standard amount may be varied depending on the level of expected contamination. Allow the added internal standard to infuse and stabilise within the fortified sample for an hour before extraction.

Add 80 g of acid modified silica gel (prepared by roller-mixing sulphuric acid and silica [63 µm to 200 µm mesh] in a ratio of 1:1.5 for min. 6 h.) to the fortified sample and blend (e.g. using an ultra-turrax™) with 200 mL dichloromethane:n-hexane (4:6), for 2 min, allow to stand for 5 min and repeat. After allowing to stand for a further 5-10 min, filter the produced slurry through silanised glass wool in a glass column or glass funnel. Wash the slurry with a further 50 mL of the extraction solvent combining the washing with the earlier filtrate. Concentrate the combined filtrate, initially to approximately 0.5 mL, then slowly (e..g. using a Reacti-therm™) to incipient dryness and solvent exchange to a methanol:water mixture (6:1, 125 µL). Add 100 µL methanol and ensure complete dissolution using vortex mixing (20 s) and ultra-sonication (5 min). Add 25 µL of water and vortex mix again for 20 s, then add 25 µL of the internal sensitivity standard (β-HBCDD-d18 in methanol) solution and mix again. If the extract turns cloudy at this stage, centrifuge prior to analysis (14,000 rpm, 1 min) and use the clear solution for measurement.

Note: A fortified in-house reference material (sunflower oil with a nominal concentration of 1-5 µg/kg of α-, β- and γ-HBCDs) may be extracted alongside the samples following the same procedure. Procedural blank controls should be included in all batches and prepared in the same way but without sample. For further confidence, all extractions may be carried out in duplicate.

5.5 Module 4 – Measurement of Extracted HBCDDs

The procedures described in this module assume competence in the technique of liquid chromatography (HPLC or UPLC) with various mass spectrometry based detection systems.

5.5.1 Module 4A –Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The equipment required for this module is a combined LC-MS/MS system consisting of:

- A high pressure liquid chromatograph equipped with an injection system, a temperature controlled separation column and a transfer line interfaced to the mass spectrometer.
- A triple quadrupole mass spectrometer equipped with negative electrospray capability, high gain detection and acquisition capacity and integrated data handling capability.
- The combined LC-MS system may be automated by the use of an auto-sampler on the LC.

Principle

Purified sample extracts containing HBCDDs are injected into the LC where the individual stereoisomers are separated on an LC column, based on rate of flow of the mobile phase and chromatographic affinity. The separated stereoisomers are transferred sequentially into the MS where they are ionised to yield characteristic ions that are separated during passage through sequentially arranged quadrupole (or hexapole) mass analysers. Ionisation products related to parent ions are detected and recorded after magnification by a detection system. The acquired signals can then be processed to yield quantitative data, either on the system or remotely by networked access.

Procedure

Extracts are analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in negative electrospray mode. Ensure that the extracts are clear before injecting 10-25 µL into the injection loop. Separation is achieved using a C18 column (2.1 x 150 mm, 1.8 µm) held at 50°C. HBCDD stereoisomers are separated using a water and methanol mobile phase in a ratio of (1:4). The programme uses isocratic elution with a flow rate of 0.5 mL/minute. Overall run-time is 8 minutes. Optionally, tetrabromobisphenol A (TBBPA) which elutes before the HBCDD stereoisomers, may also be measured in the same run, using this method.

Following transfer to the MS, HBCDD stereoisomers are ionised using negative electrospray ionisation. Typically, the following source ionisation parameters were used: fragmentor voltage 380 V, gas temperature 200°C, gas flow 20 L/min, nebuliser 30 psi, sheath gas flow 11 L/min, sheath gas temperature 350°C, capillary voltage 3000 V, nozzle voltage 1500 V, iFunnel high pressure RF 110 V, iFunnel low pressure RF 60 V. The ion transitions monitored during multiple reaction monitoring were 640.3 → 78.9 (quantification) and 640.3 → 81.1 (confirmation) and the internal standard channel was 652.3 → 78.9 and 652.3 → 80.9.The dwell time and cell accelerator voltage were set at 20 milliseconds and 2 V, respectively for all transitions. These conditions are summarised in **Table 3**.

Table 3: Typical LC-MS conditions used for HBCDD LC-MS/MS measurement

Ionisation and monitoring parameter	Specification
System used	LC-MS/MS (triple quadrupole)
Ionisation mode	Negative Electrospray
Acquisition mode	Multiple reaction monitoring
Fragmentor voltage	380 V
Argon temperature and flow rate	200 °C, 20 L/min
Nebuliser pressure	30 psi
Sheath gas temp. and flow rate	350 °C,11 L/min
capillary voltage	3000 V
Nozzle voltage	1500 V
iFunnel high and low pressure values	RF 110 V and 60 V
Monitored transitions	
Native HBCDD quantitation	640.3 → 78.9
and confirmation	640.3 → 81.1
$^{13}C_{12}$-HBCDD quantitation, confirmation	652.3 → 78.9 and 652.3 → 80.9

When the performance of the LC-MS system has been verified, the extracts can be analysed according to good QC practice. Typically, the sequence of injections should commence with the set of HBCDD calibration standards with concentrations ranging from 0.5 to 50 ng/mL. The batch of samples including a procedural blank and reference material may be run after these, followed by the duplicates, with the standards being re-run at the end of the sequence. Solvent washes or solvent blanks may also be run in between sample extracts and standards to ensure

that there is no carryover. Limits of quantitation for individual HBCDD stereoisomers may be calculated from the procedural blanks, as the average concentration of the detected analytes in the blanks plus two times the standard deviation. Typical LOQs for reliable determination should be below 0.01 µg/kg wet weight.

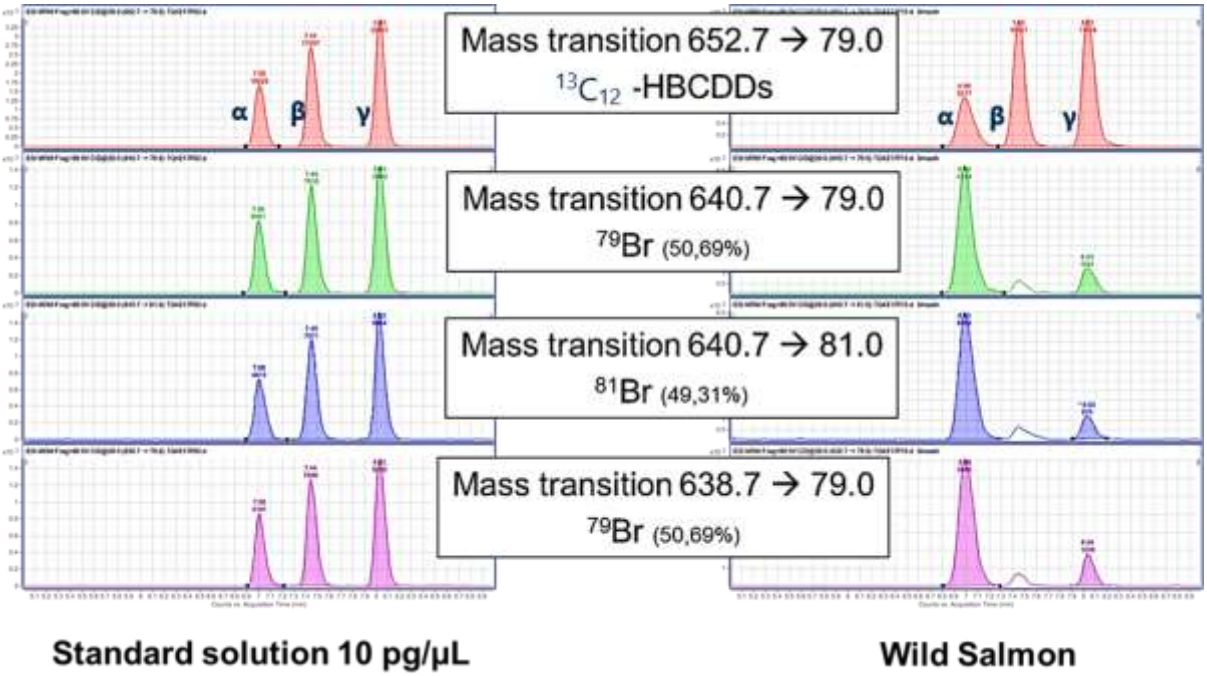

Figure 2: Example of HPLC-MS/MS chromatograms derived from a standard solution and a wild salmon sample, showing the mass transitions used for labelled and native HBCDD stereoisomers (Elution order: α-, β- and γ- HBCDD)

5.5.2 Module 4B – Liquid chromatography - Orbitrap Mass Spectrometry (LC-Orbitrap/MS)

The equipment required for this module is a combined LC-Orbitrap/MS system consisting of:

- A high pressure liquid chromatograph equipped with an auto-sampler, injection system, a temperature controlled separation column and a transfer line interfaced to the Orbitrap/MS detector.
- An Orbitrap mass spectrometer equipped with negative electrospray capability, high gain detection/acquisition capacity and integrated data handling capability.

Principle

The principle of chromatographic separation, analyte detection and data treatment is similar to that described in section 5.3.1. for the LC-MS/MS module.

Procedure

As described for LC-MS/MS, the analysis is carried out in negative electrospray ionisation mode. Chromatographic separation is achieved using a reversed-phase Kinetex-C18 (3.0 x 100 mm, 1.7 µm) column held at 40°C. The separation programme consists of a gradient elution at a flow rate of 300 µL min^{-1}, with a mobile phase consisting of (A) methanol-water (75:25, v/v) and (B) acetonitrile. The gradient is programmed as follows: the initial composition of 20:80 (A:B, v/v) is maintained for 1.0 min and then ramped to 55:45 (A:B) over 0.1 min. The gradient is held for 6.0 min at this composition and then returned to the initial conditions over 1.0 min. The column is equilibrated for 2.0 min between the runs. It is important to ensure that the extracts are clear without any suspended material before injecting 5 µL into the injection loop. The same volume is used for both, standard solutions as well as sample extracts.

Instrumental detection and measurement of the HBCDD diastereomers is performed on an Orbitrap Q Exactive mass spectrometer (Bremen, Germany) equipped with a heated electrospray ionization (HESI-II) interface operated in negative ion mode according to the settings given in **Table 4** below. Different detection modes may be used for the measurement of HBCDD diastereomers (e.g., Targeted-SIM, Full-SCAN, PRM) depending on the purpose of the analysis (e.g., improved selectivity, sensitivity or generation of wide range scan data with respect to further non-targeted treatment). Routine quantitative determination of HBCDDs is carried out in targeted selected ion monitoring (t-SIM) mode using the two most abundant ions $[M–H]^-$ of the respective molecular ion cluster for both the native and the $^{13}C_{12}$-labeled surrogates. An illustration of the expected chromatogram is given in **Figure 3**.

In some cases, apart from the typically occurring $[M–H]^-$ fragments, the recorded MS spectra may display a significant proportion of $[M+Cl]^-$ association products. In order to avoid this, measured *m/z* traces should be carefully selected during method development and rechecked if an inexplicable drop in sensitivity is noticed during routine measurement. See **Figure 3.**

Table 4: An example of ionisation conditions for the analysis of HBCDD diastereomers using LC-Negative Electrospray Orbitrap MS

Ionisation and monitoring parameter	Specification	
Sheath gas flow rate	30 a.u.	
Aux gas flow rate	6 a.u.	
Sweep gas flow rate	0 a.u.	
Spray voltage	4.00 kV	
Capillary temperature	240 °C	
S-lens RF level	50	
Aux gas heater temperature	425 °C	
Detection mode	t-SIM	
MS resolution	17 500 FWHM	
AGC target	1e5	
Maximum IT	100 ms	
Corresponding fragments and retention times:		
Compound	Quant m/z	Qual m/z
Native-HBCDDs	640.6374	638.6396
$^{13}C_{12}$-HBCDDs (ISTD)	652.6782	650.6804

In addition to the method performance, verification and QA/QC requirements described in the guidance document, an additional requirement is to ensure that the mass accuracy of measured m/z fragments are in the range of ± 5 ppm relative to the theoretical value.

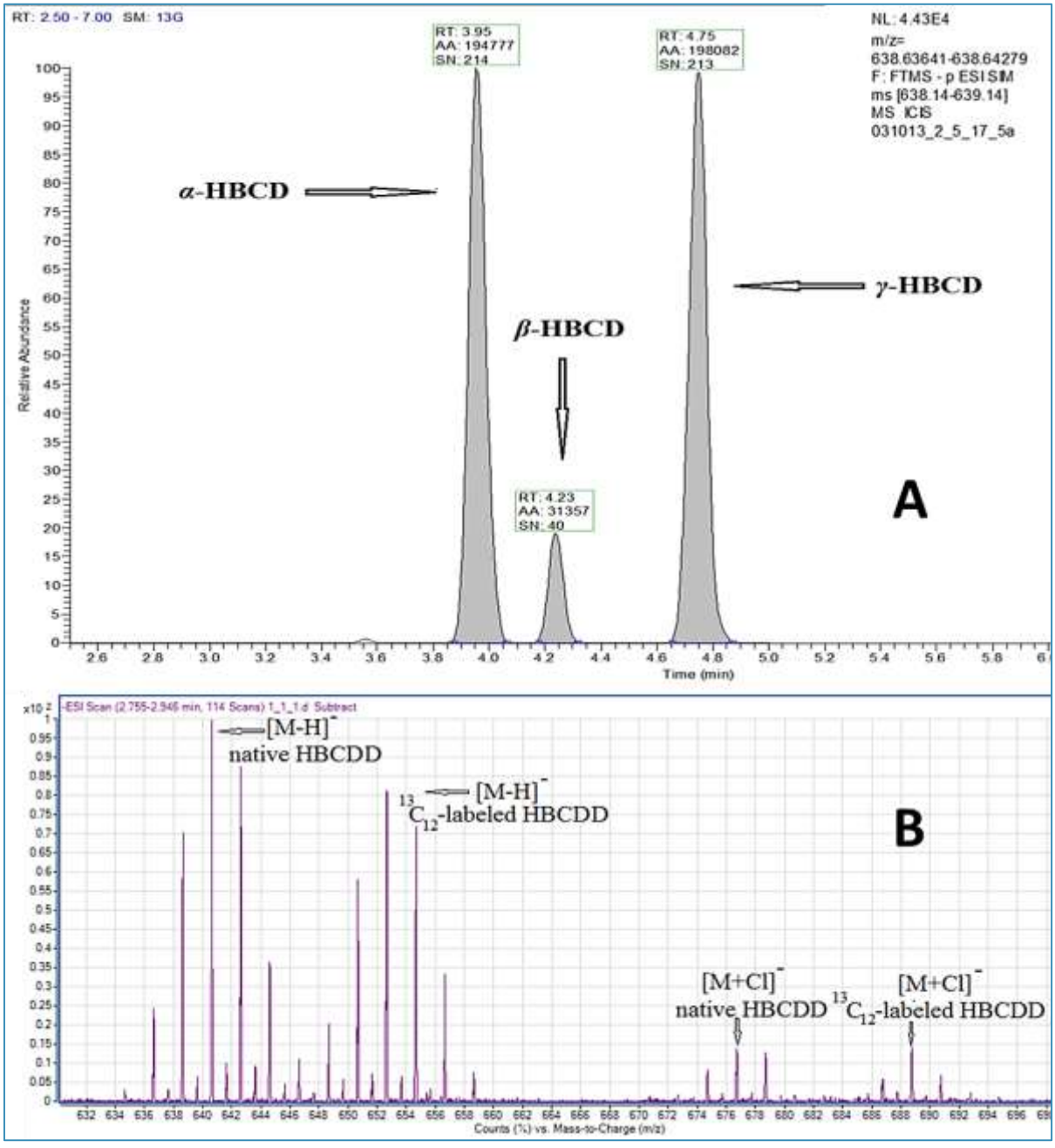

Figure 3: Trace showing indicative retention times and separation achieved for HBCDD diastereomers (A), and the MS spectra showing $[M+Cl]^-$ association products (B).

6. Quantitation and Presentation of results

Quantitation is based on internal standardisation (isotope dilution principles for those analytes with surrogate labelled internal standards), which provides a measure of the analyte content that is automatically corrected for any extraction, purification or measurement losses. The use of the labelled internal standards also allows for calculation of the recovery of the analytical process when measured against the analytical sensitivity standard.

If samples are analysed in duplicate, it is recommended that the two duplicates are measured separately and the average results is used for reporting.

HBCDD stereoisomers are identified from individual transition or ion chromatograms based on retention time of the native and corresponding labelled standard, exact mass or transition and mass ratio. These parameters should be established before quantitation. The retention times of the targeted HBCDDs should be checked and, if necessary, modified in the processing method that is usually provided with the instrument software. Use the modified method to integrate peak areas in all ion chromatograms that were produced during the analytical sequence. It is strongly recommended that the output is manually checked for correct integration.

Prepare a calibration curve encompassing the concentration range to be determined and verify the linearity of the HBCDD standard calibration for each compound, using the average responses when multiple standard runs have been carried out. If the relative response for any HBCDD stereoisomer is constant (less than 20 % coefficient of variation) over the calibration range, an averaged relative response factor (RRF) may be used for that stereoisomer. The RRF for a native HBCDD stereoisomer (i) in the analytical standard may be determined from the formula below.

$$\mathrm{RRF}_i = \left(\frac{c_{iN}}{S_{iN}}\right) \div \left(\frac{c_{iIS}}{S_{iIS}}\right) \quad (1)$$

The amount m_i in [µg] of the stereoisomer$_{(i)}$ in the sample, may be determined form the derived formula:

$$m_i = \frac{m_{IS}}{S_{iIS}} \times \frac{1}{\mathrm{RRF}_i} \times S_{iN} \quad (2)$$

m_{IS}: amount (in µg) of the internal standard added to the test sample aliquot
c_{iN}: concentration of a native HBCDD stereoisomer$_{(i)}$
c_{iIS}: concentration of the corresponding labelled HBCDD stereoisomer$_{(i)}$
S_{iN}: response (sum of two m/z's) of the native HBCDD stereoisomer$_{(i)}$
S_{iIS}: response (sum of two m/z's) of the corresponding labelled HBCDD stereoisomer$_{(i)}$

The concentration of stereoisomer c_i in the sample may then be determined by dividing the determined amount m_i in the sample in [µg] by the weight w_i in [kg] of the aliquot taken for analysis.

$$c_i = \frac{m_i}{w_i} \quad (3)$$

The quantitation software on most LC-MS systems allows the above calculation process to be automated, so that a collated list of the HBCDD concentration values for the measured sample extract is obtained.

For quality control purposes, the analytical recovery R_i of an internal standard can be calculated using equation 4 below:

$$R_i[\%] = \left(\frac{S_{iIS}}{m_{IS}}\right) \div \left(\frac{S_{RS}}{m_{RS}}\right) \times \frac{1}{RRF_{iLS}} \times 100 \quad (4)$$

m_{IS}: amount (in ng) of the internal standard$_{(i)}$ added to the test sample aliquot
m_{RS}: amount (in ng) of the internal sensitivity standard added to the test sample aliquot
S_{iIS}: response (sum of two m/z's) of the internal standard$_{(i)}$
S_{RS}: response (sum of two m/z's) of the recovery standard
$RRF_{(iIS)}$: relative response factor of the internal standard$_{(i)}$

During calibration, the coefficient of variation for any ^{13}C labelled HBCDD stereoisomer should be less than 30 %.

7. Reporting of Results

An example of a reporting format for HBCDDs concentrations in food and feed samples is given in **Table 5**.

The conventional units for reporting HBCDD concentrations are µg kg^{-1} (µg/kg) wet weight (w.w.). The concept of lower bound and upper bound sums are described in section 1.4 in the main guidance document which also provides more information on reporting in section 2.8.

Table 5: Example of a reporting format for HBCDD samples

Sample ID	
Location	
Sample Type	
Year of sampling	
Routine or Incident related	

HBCDD Stereoisomer	**Concentration [µg/kg w.w.]**	**Concentration [ng/g fat]**	MU [%]	Recovery [%]	**LOQ [µg/kg w.w.]**
α-HBCDD					
β-HBCDD					
γ-HBCDD					
σ-HBCDD					
ε-HBCDD					
Other HBCDDs					
Sum of α-, β- and γ-HBCDD					
Lower bound					
Upper bound					
Total HBCDD (by GC-MS screening)					

% Lipid content (food)	
% Moisture content (feed)	
Extraction method used	
Purification method used	
Quantitation method used	
Other relevant information	

Note: Fields in grey font are optional

8. References

[1] Annex A - Example of Methodology for the Determination of Polybrominated Diphenylethers (PBDEs) in Food and Feed of the Guidance Document Guidance Document on the Determination of Organobromine Contaminants

[2] Smedes, F. 1999. Determination of total lipid using non-chlorinated solvents. Analyst, 124, 1711–1718

[3] European Committee for Standardization (CEN), 2020. CEN Standard – EN16215:2020 - Animal feeding stuffs: Methods of sampling and analysis - Determination of dioxins and dioxin-like PCBs and of indicator PCBs by GC/HRMS.