Guidance Document on the Determination of Organobromine Contaminants

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Analytical Parameters in food and feed

EURL POPs

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Authorship

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Table of Contents

1. GENERAL

1.1. Scope and Field of Application

This document on analytical parameters for the determination of organobromine contaminants in food and animal feed was developed within the network of the European Union Reference Laboratory (EURL) for halogenated POPs in Feed and Food, the respective National Reference Laboratories (NRLs) of EU member states and international experts in the analysis of persistent organic pollutants (POPs). The guidance in this document is intended for laboratories involved in the official control of contaminants in food and feed and focuses on the determination of these substances in the laboratory. It is intended as general guidance for laboratories and particularly for those that do not have existing analytical methods. It provides guidance on the essential analytical parameters to be used for organobromine contaminant analysis in food and feed and contributes to further harmonization in this field as part of the EURL's official mandate and scope of work.

Note: All recommendations given in this document should be considered as "Guidance for reliable analyses". There are currently no regulations for the control of organobromine contaminants such as Polybrominated diphenylethers (PBDEs), Hexabromocyclododecanes (HBCDDs), etc. in food and animal feed within the EU.

The recommendations may also be applicable to the analysis of other matrices such as human tissues and environmental biota, but these are beyond the scope of this document.

Information on sampling is not given in this document. The user is referred to Commission Regulations (EC) No 152/2009 [1] and (EU) 2017/644 [2], on PCDD/Fs and PCBs as the direction given therein is expected to be similar for organobromine contaminants. Additionally, all procedures used for sampling should avoid or minimise exposure to ultra-violet radiation in order to preserve the integrity of the sample.

The recommendations contained within this guidance document apply to the different groups of organobromine contaminants, in particular to PBDEs and HBCDDs (although others are likely to be progressively included), in food and animal feed matrices.

The recommendations are designed to allow the monitoring of organobromine contaminant concentrations in food and animal feed as part of studies on:

- the establishment of current levels of these contaminants
- the establishment or maintenance of databases that may be used to recommend action levels and /or maximum levels
- the exposure assessment of populations through dietary intake and assessment of risk

Other purposes could include studies on time trends and patterns in order to identify the source(s) of possible contamination particularly during incidents involving such contamination.

1.2. Abbreviations

Abbreviation	Definition
BFR	Brominated flame retardants
CWG	Core working group
DL-PCBs	Dioxin-like polychlorinated biphenyls
eBFR	Emerging brominated flame retardants
ECD	Electron capture detection
EFSA	European Food Safety Authority
EI	Electron ionization
ESI	Electrospray ionisation
EURL	European Union Reference Laboratory
GC	Gas chromatograph(y)
HBCDDs	Hexabromocyclododecanes
HRMS	High resolution mass spectrometry
IS	Internal standard
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
LB/LB-sum	Lower bound / Lower bound sum
LOQ	Limit of quantification
LRMS	Low resolution mass spectrometry
m/z	Mass to charge ratio
MS/MS	Tandem mass spectrometry (coupling of 2 or more mass analysers)
NCI	Negative chemical ionization
NDL-PCBs	Non dioxin-like polychlorinated biphenyls
NRL	National Reference Laboratory
OFL	Official Laboratory
PBDEs	Polybrominated diphenylethers
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	Polychlorinated dibenzofurans
POP	Persistent organic pollutant
RS	Recovery standard
S/N	Signal-to-noise ratio
U	Expanded measurement uncertainty
UB/UB-sum	Upper bound / Upper bound sum
(U)HPLC	(Ultra) High performance liquid chromatograph(y)
TOF	Time-of-flight
w.w.	Wet weight

1.3. Introduction and Background

There are a number of organobromine chemicals such as polybrominated diphenylethers (PBDEs) and hexabromocyclododecanes (HBCDDs) which were produced as brominated flame retardants (BFRs). PBDEs and HBCDDs have been used in a variety of industrial and domestic applications such as transport (vehicles, trains, aircraft, etc.), plastics, furnishings, insulation, paints, electronic goods, etc. They are recognised food and animal feed contaminants, and have been listed for elimination of production and use by the Stockholm Convention [3]. Production and most applications of these organobromine chemicals are now restricted within the European Union and many other countries, although manufacture is known to continue in other parts of the world, and imported goods as well as older furnishings can still be contaminated with these chemicals. PBDEs and HBCDDs have been replaced by other BFRs that are currently produced, a number of which have been shown to be persistent, bio-accumulative and toxic in experimental studies. The legacy of earlier applications coupled with the continuing use of replacements, and the ability of these chemicals to leach out from currently used materials, ensures that they will remain as environmental and food contaminants for the foreseeable future [4].

1.4. Terms and Definitions

For purposes of this guidance document, the following terms and definitions apply.

Terms for analytical standards:

Internal standard (IS) also referred to as *'surrogate internal standards'*: a compound of known chemical purity that is added to every sample or blank in a known amount prior to any analyte extraction and/or purification steps. It is used to monitor and compensate for analyte losses during the whole sample preparation procedure and variations in instrumental sensitivity, occurring e.g. through matrix effects during ionisation (HPLC-MS/MS), adsorption and thermo-degradation in the GC inlet or variability of the volume of the extract injected into the GC. Isotope-labelled internal standards (surrogates of the analytes or closely related compounds) are used as the basis for quantification of the analytes using the isotope dilution analysis method [2].

Isotope dilution analysis method: Isotope-labelled analogues of the targeted analytes are added to the calibration standards and to each sample before extraction as internal standards in a known quantity; all analyte responses are normalised to that of the corresponding IS.

Recovery standard (RS) also referred to '*syringe/injection/volumetric standard'*: a compound of known chemical purity that is added to every sample, procedural blank or calibration standard at a known concentration, prior to instrument analysis. Recovery standards are used as basis for quantification of the IS [2].

Definitions for analytical criteria:

Apparent recovery as defined by [5] meaning the recovery derived from an analytical procedure by means of a calibration graph. In the case of isotope dilution analysis it is corrected for extraction/clean-up losses and matrix-effects and is about 100%.

Accuracy: Closeness of agreement between a test result and the accepted reference value [7]. It is determined by determining trueness and precision [8].

Limit of quantification (LOQ): Lowest content of the analyte which can be measured with reasonable statistical certainty [2].

Matrix effects (ME); interferences: The combined effects of all components of the extract other than the analyte, on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference [10]. ME can occur at various steps in the analysis, including extraction and clean-up, chromatographic separation and during detection by HPLC-MS techniques (suppression or enhancement may occur during ionisation) or by GC-MS techniques, due to absorption originating from active spots in the injector, caused in part by the presence of residual matrix compounds.

Precision: Closeness of agreement between independent test/measurement results obtained under stipulated conditions [9].

- ***Repeatability*** or 'intra-day-precision' defined as precision under repeatability conditions. Repeatability conditions means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment [8, 9]
- ***Reproducibility*** or 'inter-day-precision' defined as precision under reproducibility conditions. Reproducibility conditions means conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment [8, 9]
- ***Within-laboratory reproducibility:*** 'inter-day-precision' (intermediate precision) defined as precision under intermediate precision conditions. Intermediate precision conditions refers to conditions where measurement results are obtained with the same method, on identical test/measurement items in the same test or measurement facility, under some different operating condition [9]

Recovery or Recovery Factor as defined by [5], also referred to as 'extraction recovery' or 'absolute recovery'. This is the yield of an analyte from the extraction/clean-up stage, e.g. the recovery of the IS calculated relative to the RS.

Selectivity: The extent to which other substances interfere with the determination of a substance according to a given procedure [6].

Trueness: Closeness of agreement between the expectation of a test result or a measurement result and a true value [9]. Trueness is usually expressed as bias [8].

Lower bound: Concept which requires using zero for the contribution of each non-quantified congener to a sum parameter [2].

Upper bound: Concept which requires using the limit of quantification for the contribution of each non-quantified congener to a sum parameter [2].

1.5. Guidance for Analytical Quality Assurance

Recommendations for Laboratories

- Laboratories should be accredited by a recognised body operating in accordance with EN ISO/IEC 17011 [11] to ensure that they are applying analytical quality assurance. The methods and scope of the laboratory should be accredited to the EN ISO/IEC 17025 [12] standard.
- Laboratories should demonstrate proficiency in the analysis of organobromine contaminants at the concentration range of interest (i.e. 5 to 100 times the LOQ) by validation, ongoing internal quality control and continuous successful participation in inter-laboratory studies conducted by accredited bodies, e.g. the EURL.

General aspects regarding sample pre-treatment and storage

- The samples must be stored and transported under UV-protected conditions (e.g. using amber glassware or other suitable containment that excludes UV-radiation) in containers that can be demonstrated to be free from the relevant organobromine contaminants while preserving the integrity of the sample.
- Where required, laboratory samples should be thoroughly mixed by fine grinding, blending, etc. using a process that has been demonstrated to achieve complete homogenisation (e.g. ground to pass a 1 mm sieve for animal feed); samples may have to be dried before grinding if the moisture content is too high (The moisture content may optionally be determined for animal feed).
- The sample quantity used for the extraction should be sufficient to fulfil the requirements with respect to a sufficiently low working range including the concentrations at the suggested LOQs.
- The specific sample preparation procedures used for the products under consideration shall follow internationally accepted guidelines [13, 14].
- Appropriate measures should be taken to ensure that the sample composition is representative of the consumed food or animal feed. E.g., removal of bones, and in the case of fish, the skin has to be removed, as the contaminant content will relate to

muscle meat without skin. However, it is necessary that all remaining muscle meat and fat tissue on the inner side of the skin is carefully and completely scraped off from the skin and added to the sample to be analysed.

General aspects to avoid contamination / high blank levels

- Measures must be taken to avoid cross-contamination at each stage of the sampling and analysis procedure in the laboratory.
- Glassware should be rinsed with solvents and/or heated at temperatures suitable to remove traces of organobromine contaminants from exposed surfaces and stored under conditions preventing re-contamination.
- Reagents, glassware and other equipment used for analysis and sampling should be controlled to avoid possible introduction or loss of organobromine analytes.
- A procedural blank analysis should be performed by carrying out the entire analytical procedure omitting only the sample. The levels in the procedural blanks should be regularly monitored and maintained within an acceptable range.

1.6. Validation Parameters and General Quality Control Measures

Prior to the routine analysis of organobromine contaminants in food and feed samples, laboratories should demonstrate the performance of the methodology during the validation procedure and monitor this during routine analysis. Performance should be demonstrated in the relevant working range, with an acceptable coefficient of variation for **within-laboratory reproducibility** (see analyte specific section).

Regular procedural blanks, and fortification experiments or analysis of control samples (preferably, using reference materials or in the absence of these, materials from successful and accredited PT exercises) should be performed as internal quality control measures. Quality control (QC) charts for procedural blanks, fortification experiments or analysis of control samples should be recorded and checked to make sure that the analytical performance is in accordance with the requirements.

Table 1 provides recommendation of selected parameters for validation studies that should be carried out before routine analysis. Definitions can be found in **section 1.4**.

Table 1: Recommendations for validation studies and routine quality control measures

Grouping of matrices	▪ Use of a single food or feed matrix to represent a matrix group if the matrices in the group share similar physico-chemical properties ▪ Example for a matrix group: meat, offal, fish etc. ▪ Further sub-grouping might be necessary for analytes and/or methods if significant matrix effects are observed.
Selectivity of the analytical procedure	▪ Analytical methods should demonstrate the ability to reliably and consistently separate the analytes of interest from other co-extracted and possibly interfering compounds that may be present. ▪ Additionally, the chromatographic techniques that are used for separation/measurement should be capable of differentiation between the congeners and diastereomers of interest and other occurring congeners/diastereomers.
Analytical working range	▪ Analytical methods should demonstrate the ability to reliably and consistently measure organobromine contaminants in the range of low to sub picograms (10^{-12}g) and above.
Trueness	▪ The measurement process used must provide a valid estimate of the true concentration in a sample. This is necessary to avoid rejection of a sample analysis result on the basis of poor reliability of the determined concentration. ▪ Trueness can be estimated from regular analysis of certified reference materials, fortification experiments or participation in inter-laboratory studies.
Precision	▪ Precision can be calculated from results generated under within-laboratory repeatability and reproducibility conditions. ▪ Laboratories are expected to progress to the recording of intermediate precision, following the setting-up and training phase.
Limit of quantification	▪ LOQs are currently based on the values specified in COMMISSION RECOMMENDATION of 3 March 2014 on the monitoring of trace levels of BFRs in food (2014/118/EU) [15] and also on current levels of food and feed contamination. ▪ LOQs may be revised in the future according to evaluations resulting from new toxicological studies and hazard assessments ▪ Contaminant specific LOQs are given in the relevant sections.

2. PBDE CONGENERS

2.1. Background

Polybrominated diphenyl ethers (PBDEs) are a class of organobromine compounds that are used as additive brominated flame retardant (BFR) chemicals. PBDEs have been used in a variety of industrial and domestic applications such as construction and transport (vehicles, trains, aircraft, etc.), in materials such as plastics, furnishings, insulation, paints, electronic goods, etc. Their chemical and physical properties are similar to those of PCDD/Fs and PCBs. PBDEs constitute a group consisting of 209 possible congeners (with the degree of bromination ranging from mono- to deca-brominated; $[C_{12}H_{(10-x)}Br_xO$ (x = 1, 2, ..., 10 = m + n] as shown in figure 1), although a considerably smaller number are actually formed during commercial production. Congeners are classified according to the number of bromine atoms in the molecule. Like other POPs they are hydrophobic and highly resistant to degradation processes, particularly biodegradation, although photo degradation is known to occur. Adverse health effects have been reported for humans, animals and soil organisms. Due to their toxicity and persistence, the industrial production of technical PBDE mixtures such as “Penta-“, “Octa-“ and “Deca-BDE“ is restricted under the Stockholm convention [3].

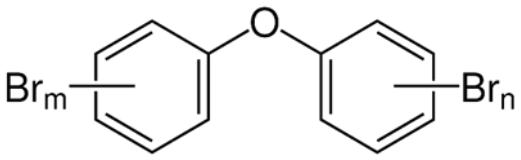

Figure 1: General PBDE structural formula

Further information on PBDEs, with respect to occurrence, toxicity and the estimation of risk through human exposure can be found in the following opinions by the European Food Safety Authority (EFSA):

Scientific Opinion on Polybrominated Diphenyl Ethers (PBDEs) in Food
https://www.efsa.europa.eu/en/efsajournal/pub/2156

2.2. Scope

The recommendations contained within **chapter 2** apply to the congener specific determination of PBDEs in food and animal feed matrices. Information on sampling is not given in this document. As the physico-chemical behaviour of these contaminants is similar to other POPs, information on these aspects that is provided in the regulations on sampling/analysis for PCDD/Fs and PCBs [13, 14] may be similarly suitable. The recommendations are designed to allow the monitoring of PBDE concentrations in food and animal feed as part of studies on monitoring, risk assessment, etc. (see General, **section 1.1**).

2.3. Analyte Definition

The requirements given below will apply to the following PBDE congeners (**Table 2**), and PBDE sum parameters in food and animal feed samples. The requirements may also be applicable to the analysis of other matrices such as human tissues and environmental biota, but these are beyond the scope of this document. Laboratories may also apply similarly appropriate conditions to the measurement of other PBDE congeners. Although BDE-138 was included in recommendation 2014/118/EU [15], reported data on food and feed over several years has consistently shown very low and often undetectable levels, so this congener was not included.

Table 2: PBDE analytes of interest

Analyte	Description	CAS Number
BDE - 28	2,2′,4-tribromodiphenyl ether	41318-75-6
BDE - 47	2,2′,4,4′-tetrabromodiphenyl ether	5436-43-1
BDE - 49	2,2′,4,5′-tetrabromodiphenyl ether	243982-82-3
BDE - 99	2,2′,4,4′,5-pentabromodiphenyl ether	60348-60-9
BDE - 100	2,2′,4,4′,6-pentabromodiphenyl ether	189084-64-8
BDE - 153	2,2′,4,4′,5,5′-hexabromodiphenyl ether	68631-49-2
BDE - 154	2,2′,4,4′,5,6′-hexabromodiphenyl ether	207122-15-4
BDE - 183	2,2′,3,4,4′,5′,6-heptabromodiphenyl ether	207122-16-5
BDE - 209	2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether	1163-19-5
Sum of 8 PBDEs without BDE-209	Sum of BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183	
Sum of 9 PBDEs including BDE-209	Sum of BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209	

2.4. Instrumentation

Gas Chromatography (GC) based analysis is recommended for PBDEs. Suitable detection methods include GC-HRMS and GC-MS/MS. Other techniques such as GC-Electron Capture Detection (GC-ECD) and GC-LRMS (both, GC-EI-MS and GC-NCI-MS) may be used if the recommended parameters, e.g., LOQ, analyte identification, etc. can be achieved.

2.5. Validation Studies and General Quality Control Measures

As described in **section 1.6**, prior to the routine analysis of PBDEs in food and feed samples, laboratories should demonstrate the performance of their methodology during the validation procedure and during routine analysis. Analytical performance should be monitored, e.g. through internal quality control measures such as regular procedural blanks, and fortification experiments or analysis of control samples (preferably, using reference materials or in the absence of these, materials from successful and accredited PT exercises).

Limit of quantification

The limits of quantification are based on the values specified in COMMISSION RECOMMENDATION of 3 March 2014, on the monitoring of trace levels of brominated flame retardants in food (2014/118/EU) [15] and also on current levels of contamination in food and feed but may be revised in the future according to evaluations resulting from new toxicological studies and hazard assessments.

For PBDE congeners, the recommended LOQ value is 0.01 µg/kg wet weight (w.w.) for individual congeners. However, given that some foods show concentrations below this level, a lower LOQ value of 0.001 µg/kg w.w. for all congeners except BDE-209 is desirable.

Since the approach chosen to estimate the LOQs has a great influence on the LOQ value, special attention should be paid to establishing this value. Approaches for the estimation of LOQs are described in the “Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food” [16]. Practically, the estimation of LOQs requires consideration of the procedural blanks as follows:

- Contribution of blank levels should be ≤ 20 % of the samples analysed in the accompanying batch
- Higher contribution requires the inclusion of blank levels in the estimation of LOQs
- Subtraction of blank concentrations may be performed, if levels remain relatively constant over time
- The variability of procedural blanks should be monitored and contributory factors (e.g. reagents, environmental conditions, etc.) should be controlled in order to reduce it.

Other validation parameters

The control of analytical recovery is essential for reliable analysis. The recovery of the added internal standards (ISs) may conveniently be measured, relative to the recovery standards (RSs). For PBDEs, except BDE-209, the recoveries of the individual internal standards should be in the range of 30 – 140 % (**Table 3**), reflecting what is currently achieved. As laboratories gain more experience in the analysis of PBDEs, the range is likely to be narrowed (e.g. 40 - 120%) based on the recoveries that are practically achievable. Lower or higher recoveries for individual congeners, may be acceptable on the condition that their contribution to the sum PBDE value does not exceed 10 % of the total.

Trueness can be estimated from regular analysis of certified reference materials, fortification experiments or by participation in inter-laboratory studies and should be between -30 % and +30 % (**Table 3**) Precision can be calculated from results generated under within-laboratory repeatability and reproducibility conditions. Within-laboratory reproducibility should not be greater than 20 % for all PBDE congeners, except BDE-209 (**Table 3**)

Table 3: Typical Performance characteristics for PBDEs

Parameter	PBDE congeners	BDE-209
Trueness	- 30 % to + 30 %	- 30 % to + 30 %
Within-laboratory reproducibility (precision)	≤ 20 %	≤ 40 %
Difference between upper and lower bound calculation[1]		
LOQ (Food)	0.01 µg/kg w.w. Target LOQ: 0.001 µg/kg w.w	0.01 µg/kg w.w.
LOQ (Feed)	0.01 µg/kg product (optionally, 12 % moisture content) Target LOQ: 0.001 µg/kg product (optionally, 12 % moisture content)	0.01 µg/kg product (optionally, 12 % moisture content)
Recovery of internal standards[2]	30 – 140 %	30 – 140 %

[1] will be specified when regulations/decisions on elevated levels are established
[2] The recovery range given is an interim measure – the range is likely to be narrowed (e.g. 40-120%) to reflect what is achievable as laboratories gain more experience.

2.6. Specific Requirements – Quantification

In the absence of regulation and based on current monitoring and literature data on food and feed occurrence, the levels for individual PBDE congeners may be considered as elevated, above a concentration of 1 µg/kg w.w. or 1 µg/kg product (optionally, 12 % moisture content) for individual congeners. This value may be lowered after the establishment of legal limits in future.

Due to the potential lability of brominated contaminants, it is recommended that internal standards (ISs) are added at the very beginning of the analytical method e.g. prior to extraction, in order to validate the analytical procedure. If the IS is added after fat extraction (e.g. for foodstuffs of animal origin containing more than 10 % fat [13]), an appropriate validation of the extraction efficiency should be carried out and verified by participation in relevant proficiency tests [18]. At least one IS congener for each relevant homologue group for PBDEs must be added, although as congener responses vary, it is preferable that all available isotope-labelled ISs of the specified PBDEs should be used as this would improve the reliability of the quantitation. If additional congeners (for which no isotope-labelled analogues are available) are being simultaneously determined, relative response factors shall be determined using appropriate isotope-labelled PBDE congeners and the validity of these can be confirmed either by using appropriate calibration solutions or by reference materials.

The use of other internal standards for methods with unit resolution MS or ECD detection is possible. For ECD, the confirmation of elevated levels through the use of two GC columns with stationary phases of different polarity, should be applied. Users of GC-ECD methods should establish that the instrumentation is capable of achieving the performance characteristics given in **Table 3**. The RS should be added just prior to instrument analysis. For MS detection, it is recommended that preferably two isotopically labelled standards are added for PBDE analysis in order to mitigate the effects of adsorption/thermo-degradation, particularly for the higher brominated compounds. Depending on the severity of adsorption/thermo-degradation, the use of a single analytical sensitivity standard may result in apparent lower or higher recoveries.

2.7. Specific Requirements – Purification and Measurement

Exclusion of interfering substances

During the clean-up process, separation of PBDE congeners from interfering matrix and other halogenated compounds should be carried out by suitable adsorption chromatography techniques (suggested effective adsorbents are silica, alumina or Florisil™).

During the gas-chromatographic separation of PBDE congeners, particular attention should be given to the separation of BDE-28, 49, 154 & 183 as co-elution of the following PBDE congener pairs has been observed during routine analysis **BDE-16/28/33**; **BDE-49/68/71**; **BB-153/BDE-154**; **BDE-175/183**. Additionally, fragments of higher brominated congeners may also cause interference. Improvement in separation is possible by using the appropriate length and phase of GC column. Parameters for checking the quality of chromatographic separation for BDE-49 and BDE-71 are also described in EPA Method 1614A [17].

Analytical calibration curve

The lower range of the calibration curve is indicated by the LOQ of 0.01 µg/kg w.w. (or targeted LOQ of 0.001 µg/kg) for each PBDE congener. The analytical range should extend to between 5.0 and 10 µg/kg, reflecting the upper ranges of the concentrations for BDE-47 and BDE-209 that are reported in the current literature. In **Table 4** selected identification requirements for a reliable PBDE analysis are listed. Elements of further identification and confirmation criteria are described in the literature [18] and in internationally recognised standards for PBDEs or similar analytes, e.g. standard EN 16215:2012 [19] or EPA methods 1614A [17] for PBDEs.

Table 4: Identification requirements for different MS techniques

GC-HRMS	
Accurate mass resolution	
Mass resolution	≥ 10 000 at 10 % valley (for the entire mass range)
Identification and confirmation of analyte signals	the retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 ions should be monitored from the same isotopic cluster
Isotope ratio	± 20 % of the theoretical value or of the corresponding reference standard
GC-MS/MS	
Unit mass resolution	
Mass resolution	Unit mass (both quadrupoles) or wider resolution for Q1 and unit mass for Q3 as established during validation
Identification and confirmation of analyte signals	the retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 transitions (including one precursor ion and one product ion each) e.g. from the same isotopic cluster should be monitored
Transition ratio[3]	± 20 % from sample extracts compared to the average of calibration standards from the same sequence
GC-LRMS	
Mass resolution	Unit mass
Identification and confirmation of analyte signals	the retention time of the targeted analyte should be -1/+2 seconds relative to corresponding standards
	2 isotopic ions should be monitored
Isotope ratio	± 20 % of the theoretical value or of the corresponding reference standard
Other identification requirements	
Target masses	See Annex A
Minimum Chromatographic separation	See section 2.7 – Exclusion of interfering substances

[3] Applying identical MS/MS conditions, in particular collision energy and collision gas pressure, for each transition of an analyte.

2.8. Reporting of Results for PBDEs

The concentrations determined in test samples should be expressed in units of µg/kg w.w. for food or in µg/kg product (optionally, relative to a feed with a moisture content of 12 %) for feed. Results should be reported to 2 significant figures. In order to facilitate comparison and to harmonise reporting, concentrations for the individual PBDE congeners shall be listed in the order of increasing IUPAC number (BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209). This listing should be followed by the two sum parameters: Sum of 8 PBDEs without BDE-209 and Sum of 9 PBDEs with BDE-209. Each of these should be given as both, lower bound (sum of congener concentrations that are ≥ the LOQ) and upper bound (sum of the above listed congener concentrations, including the LOQ value for those congeners that were below the LOQ). This will maximise the reported information and also allow interpretation of the results according to specific requirements. If additional congeners are measured, these should be listed after this ordering. An example of a reporting format for PBDEs is given in **Annex A**.

Additional information that should (optionally) be included in the report

Information on the methods used for extraction and purification for PBDEs should be included; this information can be basic, e.g. a mention of the techniques used in the analysis. If available, the lipid content should be included.

As an aid to the evaluation of the reported data, the recoveries of the individual internal standards should be included. Data for feed may also additionally be reported as µg/kg product relative to a feed with a moisture content of 12 % along with the determined moisture content, and similarly, food may additionally, also be reported on a µg/kg fat basis along with the fat content.

Measurement Uncertainty

The uncertainty of measurement for individual congeners (extrapolated to the sum parameters) should also be included as an aid to the interpretation of the data. The analytical results shall be reported as x +/– U whereby x is the analytical result and U is the expanded measurement uncertainty using a coverage factor of 2 which gives a level of confidence of approximately 95 %. The reporting of sum parameters and the possible comparison with legal limits requires the additional estimation of an expanded measurement uncertainty for theses sum parameters. For PBDEs it is the case for the sum of 8 and sum of 9 PBDEs. In these cases the calculation of the combined uncertainty u of the sum parameter is calculated as the square root of the sum of squares of the individual combined uncertainties.

As the measurement process for PBDEs is very similar to PCDD/Fs and PCBs, guidance on how to estimate the measurement uncertainty can be found in the EURL *'Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry'* [20].

3. HBCDD DIASTEREOMERS

3.1. Background

HBCDD is a type of additive BFR. It was commonly used to flame retard expanded and extruded polystyrene for use in construction, as well as in furniture upholstery, vehicle interiors and packaging material, and in other applications such as insulation, paints, electronic goods, etc. The commercial production of HBCDD generally results in the bromination pattern of 1,2,5,6,9,10-hexabromocyclododecane which has 16 possible stereoisomers. Practically, the α-, β- and γ-stereoisomers predominate in the technical product and are therefore specifically targeted in this document. Due to its persistence, toxicity, and eco-toxicity, the Stockholm Convention on Persistent Organic Pollutants decided in May 2013 to list HBCDD in Annex A to the Convention, which requires elimination of production [3].

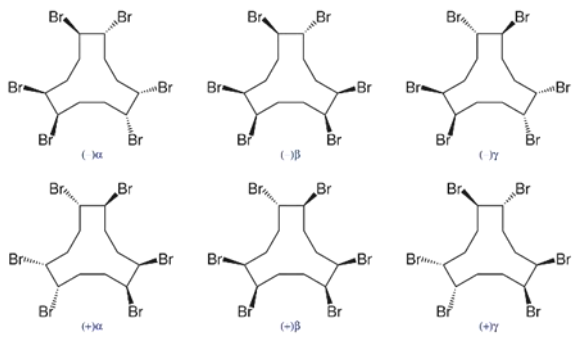

Figure 2: Structures of the three enantiomer pairs of predominantly present HBCDD stereoisomers in technical mixtures

Further information on HBCDDs, with respect to occurrence, toxicity and the estimation of risk through human exposure can be found in the following opinion by EFSA:

Scientific Opinion on Hexabromocyclododecanes (HBCDDs) in Food
https://www.efsa.europa.eu/fr/efsajournal/pub/6421

3.2. Scope

The recommendations contained within **chapter 3** of the guidance document apply to the diastereoisomer (diastereomer) specific determination of α-, β- and γ-stereoisomers of 1,2,5,6,9,10-HBCDD in food and animal feed matrices. Information on sampling is not given in this document. As the physico-chemical behaviour of these contaminants is similar to other POPs, information on these aspects that is provided in the regulations on sampling/analysis for PCDD/Fs and PCBs [13, 14] may be similarly suitable. The recommendations are designed to allow the monitoring of HBCDD concentrations in food and animal feed as part of studies on monitoring, risk assessment, etc. (see General, **section 1.1**).

3.3. Analyte Definition

The requirements given below will apply to the following HBCDD diastereomers as well as sum and total parameters in food and animal feed samples (**Table 5**). Laboratories may apply similarly appropriate conditions to the measurement of other HBCDD diastereomers.

Table 5: HBCDD analytes of interest

Analyte	Description	CAS Number
α-HBCDD	1,2,5,6,9,10-hexabromo-(1R,2R,5S,6R,9R, 10S)-rel-cyclododecane	134237-50-6
β-HBCDD	1,2,5,6,9,10-hexabromo-(1R,2S,5R,6R,9R,10S)- rel-cyclododecane	134237-51-7
γ-HBCDD	1,2,5,6,9,10-hexabromo-(1R,2R,5R,6S,9S, 10R)- rel-cyclododecane	134237-52-8
Sum of α-, β- and γ-HBCDD	Sum of 1,2,5,6,9,10-hexabromo-(1R,2R,5S,6R,9R, 10S)-rel-cyclododecane, 1,2,5,6,9,10-hexabromo-(1R,2S,5R,6R,9R,10S)- rel-cyclododecane and 1,2,5,6,9,10-hexabromo-(1R,2R,5R,6S,9S, 10R)- rel-cyclododecane	
Total HBCDD	For GC-MS based measurements. As the individual diastereomers are unresolved, the measured signal is the cumulative response of all possible HBCDD diastereomers.	

3.4. Instrumentation

High Performance Liquid Chromatography (HPLC) based analysis is recommended for the diastereomeric separation of HBCDDs in food, although GC may be used for screening total HBCDD. Techniques such as HPLC-MS/MS or HPLC-HRMS are commonly used for HBCDD analysis, although some of the instrumentation listed for PBDEs (**section 2.4**) may also be used as a screening method for total HBCDD by GC based techniques.

3.5. Validation Studies and General Quality Control Measures

As described in section 1.6, prior to the routine analysis of HBCDDs in food and feed samples, laboratories should demonstrate the performance of their methodology during the validation procedure and during routine analysis. Analytical performance should be monitored, e.g. through internal quality control measures such as regular procedural blanks, and fortification experiments or analysis of control samples (preferably, using reference materials or in the absence of these, materials from successful and accredited PT exercises).

Limit of quantification

The limits of quantification are currently based on the values specified in COMMISSION RECOMMENDATION of 3 March 2014, on the monitoring of trace levels of brominated flame retardants in food (2014/118/EU) [15], but may be revised in the future according to evaluations resulting from new toxicological studies and hazard assessments.

For HBCDD diastereomers, the recommended LOQ value is 0.01 µg/kg w.w. for food or 0.01 µg/kg product (optionally, relative to a feed with a moisture content of 12 %) for feed. For total HBCDD measured by GC-MS, the corresponding LOQ value is 0.003 µg/kg (as the cumulative response of all possible HBCDD diastereomers).

The LOQ of 0.01 µg/kg w.w. for individual diastereomers is recommended for fish and other seafood, meat and meat products, milk and dairy products, eggs and egg products, as well as infant and follow-up formula [15]. It is recognised that this LOQ may be difficult to achieve for some matrices such as animal and vegetable fats and oils, with existing instrumentation and methods, so a higher value may be more practical for these matrices as an interim measure.

Since the approach chosen to estimate the LOQs has a great influence on the LOQ value, special attention should be paid to establishing this value. Approaches for the LOQ estimation are described in the “Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food” [16]. Practically, the estimation of LOQs requires consideration of the procedural blanks as follows:

- Contribution of blank levels should be ≤ 20 % of the samples analysed in the accompanying batch
- Higher contribution requires the inclusion of blank levels in the estimation of LOQs
- Subtraction of blank concentrations may be performed, if levels remain relatively constant over time

Other validation parameters

The control of analytical recovery is essential for reliable analysis. The recovery of the added internal standards (ISs) may conveniently be measured, relative to the recovery standards (RSs). For HBCDD diastereomers, the recoveries of the individual internal standards should be in the range of 30 to 140 %, reflecting what is currently achieved. As laboratories gain more experience in the analysis of HBCDDs, the range is likely to be narrowed (e.g. 40 - 120%) based on the recoveries that are practically achievable.

Trueness can be estimated from regular analysis of certified reference materials, fortification experiments or participation in inter-laboratory studies and should be between -30 % and 30 % (**Table 6**). Precision can be calculated from results generated under within-laboratory repeatability and reproducibility conditions. Within-laboratory reproducibility should not be greater than 20 % (**Table 6**).

Table 6: Typical Performance characteristics for HBCDD

Parameter	HBCDDs
Trueness	- 30 % to + 30 % for individual diastereomers and sum
Within-laboratory reproducibility	≤ 20 % for individual diastereomers and sum
LOQ (Food) depending on matrix[4]	0.01 µg/kg w.w. for diastereomers 0.003 µg/kg w.w. for total HBCDD by GC-based screening
LOQ (Feed)	0.01 µg/kg product (optionally, at 12% moisture content) for diastereomers 0.003 µg/kg product (optionally, at 12% moisture content) for total HBCDD
Recovery of internal standards[5]	30 – 140 %

[4] fish, other seafood, meat, meat products, milk, dairy products, eggs, egg products, infant and follow-up formula. A higher value may be more practical for matrices such as fats, oils, etc., as an interim measure

[5] Recovery range given is an interim measure – the range will be narrowed (e.g. 40-120%) to reflect what is achievable as laboratories gain more experience.

3.6. Specific Requirements – Quantification

Addition of internal standards (IS) for HBCDDs must be carried out at the very beginning of the analytical method e.g. prior to extraction, in order to validate the analytical procedure. At least one isotope-labelled diastereomer (preferably, isotope-labelled α-HBCDD for the HBCDDs should be added).

For LC-MS measurements, it is preferable that isotope-labelled IS of all three specified HBCDDs should be used as this would improve the reliability of the quantitation.

If additional isomers (for which no isotope-labelled analogues are available) are being simultaneously determined, relative response factors shall be determined using appropriate isotope-labelled HBCDD diastereomers, and the validity of these can be confirmed either by using appropriate calibration solutions or by reference materials.

Due to the potential lability of brominated contaminants during the extraction and purification processes of food and feed analysis, it is necessary to add the IS to the sample aliquot prior to extraction. If the IS is added after fat extraction (e.g. for foodstuffs of animal origin containing more than 10 % fat [13], an appropriate validation of the extraction efficiency should be carried out and verified by participation in relevant proficiency tests [18].

It is also recommended that the extracts are analysed as soon as possible after reconstitution in the mobile phase used for LC-based measurements, particularly when the mobile phase contains water, as HBCDD stereoisomers may experience lower solubility. Reconstitution of the final extracts in pure methanol/acetonitrile is advisable.

It is recommended that the RS should be added just prior to HPLC- or GC-MS (HRMS, LRMS and MS/MS) analysis in order to provide the most reliable estimate of analytical recovery. While the recovery of the ISs can be calculated relative to the RS, it is possible that matrix effects may occur if extracts are not adequately purified. This may also lead to higher LOQs in matrix extracts compared to standard solutions in pure solvent. If no RS is used, a scheme for the estimation of the recovery should be included in the validation plan and appropriate experiments should be conducted for different matrices, to report the average recovery and to demonstrate that it is consistent and reproducible.

3.7. Specific Criteria – Purification and Measurement

Exclusion of interfering substances

The separation of HBCDD diastereomers from matrix interferences and other halogenated compounds should be carried out by using suitable adsorption chromatography techniques. In particular, effective fractionation of HBCDDs from any residual matrix is highly recommended in order to reduce suppression effects when HPLC-MS/MS is used for detection, as this may lead to incorrect quantitation.

Analytical calibration curve

The lower range of the calibration curve is indicated by the LOQ for HBCDD. This should extend to between 5.0 and 10 µg/kg at the higher end of range. Selected identification requirements for reliable HBCDD analysis are listed in **Table 7** and are also described in the literature [18].

Table 7: Identification requirements for HBCDDs based on commonly used MS techniques

HPLC-HRMS	
Accurate mass resolution	
Mass resolution	> 10000 at 50% FWHM
Identification and confirmation of analyte signals	the relative retention time tolerance of the targeted analyte peak and the isotope-labelled standard peak should not be greater than ± 1 %
	2 isotopic ions should be monitored from the same isotopic pattern
Isotope ratio	± 20 % of the theoretical value or of the corresponding reference standard
HPLC-MS/MS	
Unit mass resolution	
Mass resolution	Unit mass (both quadrupoles) or wider resolution for Q1 and unit mass for Q3 as established during validation
Identification and confirmation of analyte signals	the relative retention time tolerance of the targeted analyte peak and the isotope-labelled standard peak should not be greater than ±1 %
	2 transitions (including one precursor ion and one product ion each) e.g. from the same isotopic pattern should be monitored
Transition ratio[6]	± 20 % from sample extracts compared to average of calibration standards from same sequence

GC-HRMS	
Accurate mass resolution	
Mass resolution	≥ 10 000 at 10 % valley (for the entire mass range)
Identification and confirmation of analyte signals	the retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 ions should be monitored from the same isotopic cluster
Isotope ratio	± 20 % of the theoretical value or of the corresponding reference standard
GC-MS/MS	
Unit mass resolution	
Mass resolution	Unit mass (both quadrupoles) or wider resolution for Q1 and unit mass for Q3 as established during validation
Identification and confirmation of analyte signals	the retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 transitions (including one precursor ion and one product ion each) e.g. from the same isotopic cluster should be monitored
Transition ratio[6]	± 20 % from sample extracts compared to the average of calibration standards from the same sequence
Other identification requirements	
Target masses	See Annex A
Minimum Chromatographic separation	See section 2.7 – Exclusion of interfering substances

[6] applying identical MS/MS conditions, in particular collision energy and collision gas pressure, for each transition of an analyte

3.8. Reporting of Results for HBCDDs

The concentrations determined in test samples should be expressed in units of µg/kg w.w. for food or in µg/kg product (optionally, relative to a feed with a moisture content of 12 %) for feed. Results should be reported to 2 significant figures. In order to facilitate comparison and to harmonise reporting, concentrations for the individual HBCDD diastereomers should be listed in the conventional order i.e. α-HBCDD, β-HBCDD and γ-HBCDD. This listing should be followed by the sum parameter "Sum of α-, β- and γ- HBCDD", which should be given as both, lower bound (sum of diastereomer concentrations that are ≥ the LOQ) and upper bound (sum of the above listed diastereomer concentrations, including the LOQ value for those diastereomers that were below the LOQ). This will maximise the reported information and also allow interpretation of the results according to specific requirements. If additional diastereomers are measured, these should be listed after this ordering. Measurements that are carried out by GC-MS methods (screening) will report only a single concentration of total HBCDD. An example of a reporting format for HBCDDs is given in **Annex B**.

Additional information that should (optionally) be included in the report

Information on the methods used for the extraction and purification of HBCDDs should be included – this information can be basic, e.g. a mention of the techniques used in the analysis. If available, the lipid content should be included.

As an aid to the evaluation of the reported data, the recoveries of the individual internal standards should be included. Data for feed may also additionally be reported as µg/kg product relative to a feed with a moisture content of 12 % along with the determined moisture content, and similarly, food may also be reported on a µg/kg fat basis along with the fat content.

Measurement Uncertainty

The uncertainty of measurement for individual diastereomers (extrapolated to the sum parameter) should also be included as an aid to the interpretation of the data. The analytical results shall be reported as x +/– U whereby x is the analytical result and U is the expanded measurement uncertainty using a coverage factor of 2 which gives a level of confidence of approximately 95 %. The reporting of sum parameters and the possible comparison with legal limits requires the additional estimation of an expanded measurement uncertainty for theses sum parameters. For HBCDDs it is the case for the sum of α-, β- and γ- HBCDD. In these cases the calculation of the combined uncertainty u of the sum parameter is calculated as the square root of the sum of squares of the individual combined uncertainties. As the principle of the measurement process for HBCDDs is similar to PCDD/Fs and PCBs, guidance on how to estimate the measurement uncertainty can be found in the EURL *'Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry'* [20]

4. LITERATURE

[1] COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed

[2] Commission Regulation (EU) 2017/644 of 5 April 2017 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014

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[13] COMMISSION REGULATION (EU) 2017/644 of 5 April 2017 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014

[14] COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed

[15] European Commission (EC), 2014. Commission Recommendation 2014/118/EU of 3 March 2014 on the monitoring of traces of brominated flame retardants in food. Official Journal of the European Union, L65/39, 5.3.2014.

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5. ANNEXES

The following annexes provide examples of the analytical procedures that have been used successfully by experienced NRLs and may be helpful for those laboratories that are new to organobromine contaminant analysis. Annexes for other organobromine contaminants may be added in the future.

ANNEX A: Example of Methodology for the Determination of Polybrominated diphenylethers (PBDEs) in Food and Feed

Published 20 April 2022

(Please double click on the pdf-icons to open the ANNEX A)

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

Published 10 May 2023

(Please double click on the pdf-icons to open the ANNEX B)