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Determination of Hexabromocyclododecane
(HBCD) in sediment and biota

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Our cover photo was taken by N. Penny Holliday aboard the RRS “Discovery” in rough seas in the Rockall Trough.

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Abstract

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This document provides advice on the analysis of hexabromocyclododecane (HBCD) in sediment and biota. The analysis of HBCD in sediment and biota generally involves extraction with organic solvents, clean-up, and either gas chromatographic separation with mass-spectrometric (MS) detection or liquid chromatography with MS detection. All stages of the procedure are susceptible to insufficient recovery and/or contamination. Where possible, quality-control procedures are recommended to check the method's performance. These guidelines are intended to encourage and assist analytical chemists to reconsider their methods and to improve their procedures and/or the associated quality-control measures where necessary.

Keywords: hexabromocyclododecane, sediment, biota, sample pretreatment, storage, extraction, clean-up, calibration, liquid chromatography, mass spectrometry.

1 Introduction

Hexabromocyclododecane (HBCD) is produced by the bromination of cyclododec-1,5,9-triene and has been used since the late 1970s. HBCD is an additive flame retardant, used predominately in expanded and extruded polystyrene for thermal-insulation foams that are used in building and construction and in textile back-coatings. HBCD can be released to the environment during its production, while manufacturing other products to which HBCD is being added, or during disposal of products containing this chemical. In addition, HBCD may continue to leak from treated material and constitute a diffuse source of this compound to the environment. Atmospheric transportation is thought to be a major pathway for HBCD into the marine environment; point sources may also exist. HBCD has been found in remote areas of Sweden and Finland and in the Arctic (de Wit *et al.*, 2006).

Theoretically, there are 16 possible stereoisomers of HBCD: six diastereomeric pairs of enantiomers and four mesoforms. However, in technical HBCD mixtures, mainly three of the six enantiomeric pairs are found, namely α -, β -, and γ -HBCD, with the dominant isomer being γ -HBCD (Law *et al.*, 2005; Figure 1). In sediment, the γ -isomer also dominates, but in biota, the major isomer is α -HBCD (Law *et al.*, 2005). β -HBCD is always a minor component. HBCD has a relatively high octanol water-partition coefficient, $\text{Log } K_{ow} = 5.8$, and it has the potential to bioaccumulate. The hydrophobic nature of HBCD means that it will associate with particulate material and will accumulate in sediment, particularly if the sediment has high organic-carbon content. A bioconcentration factor (BCF) of $18\,000\text{ kg}^{-1}$ has been estimated (OSPAR, 2001). It is thought to be persistent; however, there is very little information available on the persistence of this compound in the marine environment. The properties of persistence in the environment, its tendency to bioaccumulate, and its toxicity to aquatic and terrestrial organisms have not been fully characterized. The risks to human health have not been fully evaluated. HBCD has been shown to have sublethal effects on juvenile rainbow trout (Ronisz *et al.*, 2001). Induction of catylase activity and antagonistic effects on CYP1A and an increase in the liver somatic index were observed after five days of exposure of juvenile trout to HBCD. Developmental neurotoxic effects have also been observed in neonatal mice (Eriksson *et al.*, 2002). Limited endocrine disruption of the reproductive and thyroid hormonal systems have been observed in juvenile flounder at HBCD concentrations of up to $446\ \mu\text{g g}^{-1}$ lipid weight in flounder muscle (Kuiper *et al.*, 2007).

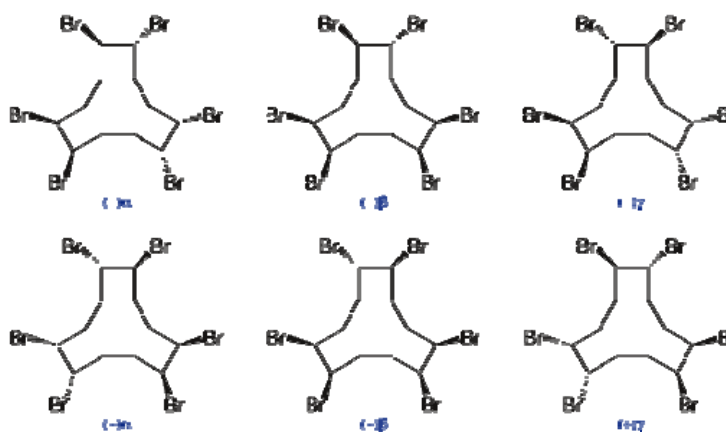


Figure 1. Structures of the six (out of 16 possible) hexabromocyclododecane isomers that are present in the technical product at $>1\%$ of total HBCDs.

2 Appropriate species for analysis of HBCD

For the Northeast Atlantic, guidance on the selection of appropriate species for contaminant monitoring is given in the OSPAR Joint Assessment and Monitoring Programme (JAMP) guidelines (OSPAR Commission, 2002). Other species, such as sole (*Solea solea*), hake (*Merluccius merluccius*), and oysters (*Ostrea* sp. or *Crassostrea* sp.), may also be appropriate. The HELCOM COMBINE Manual provides advice on appropriate species for the Baltic Sea¹. Existing data indicate that HBCD concentrations for shellfish are very low, therefore detecting long-term trends may be difficult using these species. High trophic-level organisms and lipid-rich tissue will accumulate higher levels of HBCD, possibly making them more suitable for temporal-trend monitoring.

3 Pretreatment and storage

3.1 Contamination

Sample contamination could occur during sampling, sample handling, pretreatment, and analysis, and may be caused by the environment, the containers or packing materials used, the instruments used during sample preparation, or may stem from the equipment, solvents, and reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of fish on board ship. Background contamination is not as important for HBCD as for polybrominated diphenyl ethers (PBDE). However, it remains important that the likely sources of contamination are identified and steps taken to avoid sample handling in areas where contamination could occur. One way of minimizing the risk is to conduct dissection in a clean area, such as within a laminar-flow hood away from the deck areas of the vessel, and to avoid contact with potentially HBCD-containing materials, such as packing materials.

Plastic materials, except polyethylene or polytetrafluorethene, must not be used for sampling, because of the possible adsorption of contaminants onto such materials. Samples should be stored in solvent-washed aluminium cans or glass jars (preferably prebaked at 450 °C). Aluminium cans are better, because glass jars are more susceptible to breakage. Sediment samples should be transported in closed containers; a temperature of 25 °C should not be exceeded. If samples are not analysed within 48 h after sampling, they must be stored for the short term at 4 °C. Storage over several months should be limited to frozen (<-20 °C) and dried samples. Biota samples should be kept cool or frozen (<-20 °C) as soon as possible after collection. Live mussels should be transported in closed containers at temperatures between 5 °C and 10 °C.

3.2 Shellfish

3.2.1 Depuration

Depending upon the situation, it might be desirable to depurate shellfish to void the gut contents and any associated contaminants before freezing or sample preparation. Samples collected close to point sources are usually depurated. The gut contents of these shellfish may have significant quantities of HBCD associated with food and

¹ http://www.helcom.fi/groups/monas/CombineManual/en_GB/main/

sediment particles that have not been assimilated into the tissues of the mussels. Depuration should be undertaken in controlled conditions and in filtered water taken from the sampling site; depuration over a period of 24 h is usually sufficient. The aquarium should be aerated and temperature controlled.

3.2.2 Dissection and storage

Mussels should be shucked live and opened with minimal tissue damage, by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed, homogenized as soon as possible, then frozen at -20°C in solvent-washed glass jars, preferably prebaked at 450°C , or aluminium tins, until analysis.

When samples are processed, both at sea and on shore, the dissection must be undertaken by trained personnel on a clean bench, wearing clean gloves, and using clean stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment, such as homogenizers, should be cleaned by wiping down with tissue and washing with an appropriate solvent. Knives should only be sharpened using steel, to prevent contamination of the blade from the oils used to lubricate sharpening blocks.

3.3 Fish

3.3.1 Dissection and storage

Ungutted fish should be wrapped individually in suitable material (e.g. solvent-washed aluminium foil) and stored at $<-20^{\circ}\text{C}$. If plastic bags or boxes are used, they should be used only as outer containers and should not come into contact with tissues. Organ samples, for example, livers, should be stored in solvent-washed containers made of glass, stainless steel, or aluminium, or should be wrapped in precleaned aluminium foil. The individual samples should be clearly and indelibly labelled and stored together in a suitable container at a temperature of -20°C until analysis. If the samples are to be transported during this period (e.g. from the ship to the laboratory), arrangements must be made to ensure that the samples do not thaw during transport.

When samples are processed, both at sea and on shore, the dissection must be undertaken by trained personnel wearing clean gloves and using solvent-washed stainless steel knives and scalpels on a bench previously washed with detergent (e.g. Decon 90). Stainless steel tweezers are recommended for holding tissue during dissection. After the samples have been prepared, all tools and equipment (such as homogenizers) should be cleaned by wiping with tissue and rinsing with solvent.

3.3.2 Subsampling

When sampling fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. To ensure uniformity, the right-side dorso-lateral muscle should be sampled. If possible, the entire right-side dorso-lateral fillet should be homogenized and subsamples taken for replicate HBCD determinations. If, however, the amount of material to be homogenized is too large, a specific portion of the dorsal muscle should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin be used in this case.

When dissecting the liver, care should be taken to avoid contamination from the other organs. If bile samples are to be taken, they should be collected first. If the whole liver is not to be homogenized, a specific portion should be chosen to ensure comparability. When pooling of tissues is necessary, an equivalent quantity of tissue should be taken from each fish, for example, 10% from each whole fillet.

3.4 Sediment

HBCD should be extracted from dried samples, because storage, homogenization, and extraction are much easier when the sediment is dry. Chemical drying can be done by grinding with Na_2SO_4 or MgSO_4 until the sample reaches a free-flowing consistency. It is essential that at least several hours elapse between grinding and extraction to allow for complete dehydration of the sample; residual water might decrease extraction efficiency. Freeze-drying is a popular technique for drying sediment; however, possible losses or contamination must be checked. Losses through evaporation are diminished by keeping the temperature in the evaporation chamber below 0 °C. Contamination during freeze-drying is reduced by putting a lid, with a hole of ca. 3 mm in diameter, on the sample container. Before taking a subsample for analysis, the samples should be thoroughly mixed using a metal spatula. Freeze-dried samples should be stored at room temperature and wet sediment frozen at -20 °C or below. More information is provided in the JAMP guidelines for monitoring contaminants in sediment (OSPAR Commission, 2002).

4 Analysis

4.1 Solvent purity and blanks

For work at low concentrations, the use of high-purity solvents is essential, particularly when large solvent volumes are being used for extraction and column clean-up. All batches of solvents should be checked for purity by concentration of an aliquot of solvent by at least the same volume factor as used in the overall analytical procedure. Batches that demonstrate significant contamination, which will interfere with the analysis, should be rejected.

4.2 Preparation of materials

In addition to solvents, reagents and adsorptive materials must be free of HBCD and other interfering compounds. Therefore, precleaning of all reagents, for example, alumina, silica, sodium sulphate, hydromatrix, etc., is essential, either by solvent extraction and/or by heating in a muffle oven as appropriate. Glassfibre materials, for example, Soxhlet thimbles and filter papers used in pressurized liquid extraction (PLE), should be cleaned by solvent extraction or prebaked at 450 °C overnight. It should be borne in mind that clean materials can be recontaminated by exposure to laboratory air and, therefore, the method of storage after cleaning is critical. Ideally, materials should be prepared immediately before use, but if they are to be stored, the conditions should be considered carefully. All containers that come into contact with the sample should be made of glass or aluminium and should be precleaned before use.

4.3 Lipid determination

The lipid content of tissues can be useful in characterizing the samples and reporting concentrations in biota on a wet-weight or lipid-weight basis. The total lipid content of fish or shellfish should be determined using the method of Bligh and Dyer (1959), as modified by Hanson and Olley (1963), or an equivalent method, such as Smedes (1999). Extractable lipid may be used, particularly if the sample size is small and lipid content is high. It has been shown that, if the lipid content is high (>5%), this will be comparable with the total lipid. If extraction techniques are applied that destroy or remove lipid materials, for example, PLE with fat retainers, the lipid content should be determined on a separate subsample of the tissue homogenate.

4.4 Dry weight determination

The dry weight of shellfish or sediment samples should be determined gravimetrically, so that concentrations can also be expressed on a dry-weight basis.

4.5 Extraction and clean-up

HBCD is hydrophobic and will have an affinity for particles; therefore, they can potentially accumulate in sediments, particularly those sediments with a high organic carbon. It can also concentrate in the lipids of an organism. HBCD can be extracted using techniques used for other lipophilic, non-polar compounds, such as CBs and PBDEs (Morris *et al.*, 2006). A range of methods has been used for the extraction of HBCD from biota and sediment. These include the more traditional methods, such as Soxhlet extraction or Ultra Turrax homogenization, and newer automated methods, such as pressurized liquid extraction (PLE). For Soxhlet extractions, hexane/acetone mixtures are commonly used combined with an extraction time of between 6 h and

24 h. Hexane/acetone mixtures are also used with PLE, if no fat retainers are used, with an extraction time of ~10 min per sample. PLE or Soxhlet extraction are therefore the preferred methods, with PLE having the advantage of using less solvent, being fully automated and taking less time.

Tissue and sediment extracts will always contain many compounds other than HBCD. A suitable clean-up is necessary to remove the compounds that may interfere with the subsequent analysis. Different techniques may be used, either singly or in combination, the choice being influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method used. If Soxhlet extraction is used for biota, there is a much greater quantity of residual lipid to be removed before the analytical determination can be made. The most commonly used clean-up methods involve the use of alumina- or silica-adsorption chromatography, but gel-permeation chromatography (GPC) can also be used. For GPC, two serial columns are often used for improved lipid separation. Either dichloromethane or solvent mixtures, such as dichloromethane/hexane or cyclohexane/ethyl acetate, can be used as eluents for GPC. Depending on the detection method and the lipid content of the sample, it may be necessary to use a second clean-up step to separate HBCD from other interfering compounds. HBCD is stable under acid conditions; therefore, treatment with sulphuric acid or acid-impregnated silica columns may be used in the clean-up.

One advantage of using PLE is the possibility of combining the clean-up with the extraction; this is especially useful where mass spectrometry is being used as the detection method. Methods have been developed for online clean-up and fractionation of dioxins, furans, and PCBs with PLE for food, feed, and environmental samples (Sporring *et al.*, 2003). The methods use a fat retainer for the online clean-up of fat. Silica impregnated with sulphuric acid, alumina, and Florisil has been used as fat retainers. A non-polar extraction solvent, such as hexane, should be used, if fat retainers are used during PLE. This method can also be applied to the extraction of HBCD. However, if tetrabromobisphenol A (TBBP-A) is also to be extracted, this method is not feasible, because of retention of this compound on the fat retainer.

4.6 Preconcentration

TurboVap sample concentrators can be used to reduce solvent volume. Using rotary-film evaporators is more time-consuming but more controllable. Buchi Syncore Analyst systems are also more controllable and are as rapid as TurboVap concentrators and have the advantage of automatically rinsing down the sides of the vial, if a flush-back module is fitted while concentrating. In contrast to PBDEs and CBs, where the evaporation steps have to be carefully optimized to avoid losses of the lower brominated or chlorinated compounds, loss of HBCD during concentrations is not an issue. When reducing the sample to a final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the gas chromatograph (GC) include pentane, hexane, heptane, and isooctane. For analysis by LC-MS, samples are usually taken to dryness and reconstituted in methanol.

4.7 Instrumental determination of HBCD

Analysis of HBCD is less straightforward than the analyses of PBDEs, and a different approach is usually required. HBCD can be determined by gas chromatography-mass spectrometry (GC-MS), but the analysis can be problematic. The uncertainty is greater than for PBDEs analysed using the same method (Covaci *et al.*, 2003). GC-

electron-capture detection (ECD) is not recommended, because of the limited linear range and lack of selectivity. Moreover, if GC-ECD is used, the clean-up will have to separate out all other organohalogenated compounds that may give coelution problems. In addition, the three main HBCD diastereoisomers found in technical mixtures cannot be separated by GC; only a total concentration can be determined. A liquid-chromatography (LC) method using a chiral HPLC column is required to separate the three diastereoisomers, with separation of enantiomers being possible.

For analysis by both GC-MS and LC-MS, automatic sample injection should be used wherever possible, to improve the reproducibility of injection and the precision of the overall method.

4.7.1 Gas chromatograph - mass spectrometry

Few publications report the analysis of HBCD along with the PBDEs by GC-MS, although it has been done using both GC-electron-capture negative ionization (ECNI) and high-resolution GC-MS. Splitless, pulsed-splitless, programmed temperature vaporizer (PTV), and on-column injectors have been used for the determination of HBCD. Automatic sample injection should be used wherever possible, to improve the reproducibility of injection and the precision of the overall method. Mainly non-polar columns are used, for example, HT-8, DB-5, and STX-500. Both high- and low-resolution GC-MS can be used in conjunction with either electron ionization (EI) or ECNI. ECNI provides more sensitivity than EI or positive-impact chemical ionization (PCI). Either ammonia or methane may be used as the reagent gas when using chemical ionization. When GC-ECNI-MS is used, the bromine ion only is monitored; therefore, isotopically labelled standards (^{13}C) cannot be used as internal standards for quantification purposes. Larger-fragment ions, required for structural confirmation, are not formed in ECNI mode.

HBCD isomers interconvert at temperatures $>160\text{ }^{\circ}\text{C}$; therefore, the three HBCD diastereoisomers cannot be separated, and a broad hump is obtained in the GC chromatogram. In addition, the three diastereoisomers will have different response factors; therefore, the concentration of HBCD cannot be determined accurately by GC-MS (de Boer and Wells, 2006). Moreover, HBCD degrades at $240\text{ }^{\circ}\text{C}$, therefore there may be significant losses of HBCD during GC analysis. Cold on-column injection, short GC columns, and thin, stationary films can minimize the degradation of HBCD. When analysing for HBCD by GC-MS, the liner should be changed after each batch of samples to keep it as clean as possible. Coelution of HBCD with certain PBDEs can also be a problem.

4.7.2 Liquid chromatography - mass spectrometry

A reverse-phase column should be used for analysis of HBCD by LC-MS. The three diastereoisomers found in the technical mixture should separate easily using a column, such as a C_{18} and either methanol/water or acetonitrile/water, usually with ammonium acetate (10 mM) as the mobile phase. Typically, the flow rate will be $\sim 250\text{ }\mu\text{l min}^{-1}$, and a gradient programme will be required. HPLC with chiral columns, such as permethylated β -cyclodextrin columns, can also be used to separate the enantiomers of the α , β , and γ -HBCD diastereoisomers. Either electrospray or atmospheric-pressure chemical-ionization (APCI) can be used. However, electrospray is more sensitive and is therefore recommended. Clean-up of the samples before analysis is important, to avoid matrix effects and ion suppression. The deprotonated molecular ion ($m/z = 640.7$) should be the major ion, fragment ions may also be identified to be used as qualifier ions. LC-MS has poorer detection limits than GC-

MS, with the sensitivity being approximately ten times less than that of the GC-NCIMS method. Using LC-MS, and with an injection volume of $\sim 15 \mu\text{l}$, it should be possible to detect $\sim 0.5 \text{ ng}$ on-column (Morris *et al.*, 2004). LC-MS-MS can usually overcome the problem of higher detection limits.

5 Calibration and quantification

5.1 Standards

HBCD standard solutions for each of the three major stereoisomers, α -, β -, and γ -HBCD, of known purity should be used for the preparation of calibration standards. If the quality of the standard materials is not guaranteed by the producer or supplier, as for certified reference materials, it should be checked by GC-MS analysis. Calibration standards should be stored in sealed, amber glass ampoules. Otherwise, they can be stored in a refrigerator in stoppered measuring cylinders or flasks that are gas tight to avoid evaporation of the solvent during storage.

Ideally, internal standards should fall within the concentration range of the compounds to be determined and should not include compounds that may be present in the samples. Deuterated and ^{13}C -labelled HBCD standards are available for the three major diastereoisomers for use as internal standards in HBCD analysis using GC-EIMS or LC-MS. However, deuterated standards are less expensive and are therefore the preferred option. Because HBCD is prone to ion suppression, it is recommended that a labelled standard should be used for each isomer being analysed by LC-MS. When using GC-ECNI-MS, these are of little value, because only the bromine ions can be monitored. When GC-ECNI-MS is used for the analysis, a recovery standard should be added to each sample before extraction and the recovery calculated as a check on the method.

5.2 Calibration

Multilevel calibration with at least five calibration levels is preferred to define the calibration curve adequately. In general, GC-MS calibration is linear over a considerable concentration range, but exhibits non-linear behaviour when the mass of a compound injected is low because of adsorption. Quantification should be conducted in the linear region of the calibration curve, or the non-linear region must be well characterized during the calibration procedure. External standardization is used for HBCD with GC-ECNI-MS, because only the bromine ions are monitored. An internal standard method should be used when GC-EIMS or LC-MS is used.

6 Analytical quality control

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination that they consider acceptable. Achievable limits of determination, based on a 10 g sample² for HBCD, on a wet-weight basis for biota and on a dry-weight basis for sediment are:

- for GC-ECNI-MS: $0.05 \mu\text{g kg}^{-1}$ for the sum of the four diastereoisomers,
- for LC-MS: $0.5 \mu\text{g kg}^{-1}$ for each diastereoisomer,
- for LC-MS/MS: $0.05 \mu\text{g kg}^{-1}$ for each diastereoisomer.

A procedural blank should be measured with each batch of samples and should be prepared simultaneously using the same chemical reagents and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will result in errors in quantification. The procedural blank is also very

² Detection limits will be higher if smaller sample sizes are used.

important in the calculation of limits of detection and limits of quantification for the analytical method. For GC-EIMS or LC-MS analysis, labelled standards can be added after or before extraction; those from which the absolute recovery will be assessed are added before GC-MS injection. This ensures that the calculated HBCD concentrations are corrected for the recovery obtained in each case. For GC-ECNI-MS, recovery of HBCD should be checked and reported. In the case of GC-ECNI-MS, a recovery standard, such as CB198, should be added before extraction and the recovery calculated for each sample by reference to an external standard. Recoveries should be between 70% and 120%; if not, samples should be repeated. In addition, a laboratory reference material (LRM) should be analysed within each sample batch if available. The LRM must be homogeneous and well characterized for the determinands of interest within the analytical laboratory. Ideally, the LRM should be of the same matrix type, for example, sediment, liver, muscle, mussel tissue, because the samples and the determinand concentrations should be in the same range as those in the samples. The data produced for the LRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM in duplicate from time to time, to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision. Currently, there are no certified reference materials available for HBCD in sediment or biota. At regular intervals, the laboratory should participate in an intercomparison or proficiency exercise where samples are circulated without knowledge of the determinand concentrations, to provide an independent check on performance.

7 Data reporting

The calculation of results and the reporting of data can represent major sources of error. Control procedures should be established to ensure that data are correct and to obviate transcription errors. Data stored in databases should be checked and validated, and checks are also necessary when data are transferred between databases. Where possible, data should be reported in accordance with the latest ICES reporting formats.

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9 Abbreviations and technical terminology

APCI	atmospheric-pressure chemical-ionization
BCF	bioconcentration factor
CB	chlorobiphenyl
CRM	certified reference material
CYP1A	cytochrome P450
ECD	electron-capture detection
ECNI	electron-capture negative ionization
EI	electron ionization
GC	gas chromatography
GPC	gel-permeation chromatography
HBDC	hexabromocyclododecane
HPLC	high performance liquid chromatography
LC	liquid chromatography
LRM	laboratory reference material
MS	mass spectrometry
NCIMS	negative chemical ionization mass spectrometry
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCI	positive-impact chemical ionization
PLE	pressurized liquid extraction
PTV	programmed temperature vaporizer
TBBP-A	tetrabromobisphenol A

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