A REVIEW OF HEXABROMOCYCLODODECANE (HBCD) AND TETRABROMOBISPHENOL-A (TBBP-A) IN THE AQUATIC ENVIRONMENT AND THE DEVELOPMENT OF AN ANALYTICAL TECHNIQUE FOR THEIR ANALYSIS IN ENVIRONMENTAL SAMPLES

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December 2007
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SUMMARY

1. Due to their persistence, potential for long-range atmospheric transport, high bioaccumulation and toxicity, brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBP-A) are listed on the Oslo and Paris Commission (OSPAR) List of Chemicals for Priority Action. Furthermore PBDEs and HBCD are included on the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP) and although currently classed as pre-CEMP determinands (voluntary) are likely to be reclassified as mandatory determinands.

2. BFRs have been found in the OSPAR Convention area in sediment and fish and in areas remote from known sources, indicating they are widespread environmental contaminants.

3. Although not produced in Scotland, PBDEs and HBCD have been produced within the UK and have been detected in the UK marine environment.

4. A method for the analysis of PBDEs was recently developed at FRS and environmental samples from a range of locations around Scotland were analysed for PBDEs. PBDEs were found in both biota and sediment from the Scottish aquatic environment. However, data on other BFRs, such as HBCD and TBBP-A, is scarce. There is a requirement to establish if these BFRs are also present in the Scottish aquatic environment.

5. Analytical methods for the analysis of HBCD and TBBP-A were developed for biota at FRS ML. Both methods involved the Pressurised Liquid Extraction (PLE) of HBCD and TBBP-A from biota using iso-hexane. Samples were cleaned up by the addition of 5% deactivated alumina (HBCD only) to the PLE extraction tubes followed by gel permeation chromatography (GPC). The solvent was removed by rotary evaporation and the residue reconstituted in methanol prior to analysis by liquid chromatography-mass spectrometry (LC-MS) using electrospray ionisation. An internal standard method, using labelled standards, was used for quantification.

6. This method was fully validated for the analysis of HBCD diastereoisomers and TBBP-A in biota. Replicate analysis of the high and low standards on separate days by LC-MS gave CV% of <10%. Recoveries were >90% for biota.
Limits of detection (LoDs) for HBCD and TBBP-A were around 0.3 μg kg⁻¹ wet weight for mussels or fish muscle (10 g sample extracted) and 6 μg kg⁻¹ wet weight fish liver (0.5 g fish liver extracted).

INTRODUCTION

Brominated flame retardants (BFRs) include polybrominated diphenyl ethers (PBDE), hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBP-A). BFRs are used to reduce fire hazards by interfering with the combustion of polymeric materials and can be classed as additive or reactive materials. Reactive BFRs are chemically bonded into plastics. Additives, such as PBDEs and HBCD, are added to polymers and resins and are thought to be more easily released to the environment when compared to reactive BFRs. TBBP-A is used as both a reactive and an additive BFR.

PBDEs and HBCD were produced in the UK. Penta and octa formulations of PBDEs were manufactured by the Great Lakes Chemical Company at Newton Aycliffe, County Durham in the north east of England. Manufacture of PBDEs at this plant ceased in 1996 and was replaced with HBCD. The plant finally closed in December 2003. BFRs can be released to the environment during their production and while manufacturing other products, and during disposal of products containing these chemicals. In addition, BFRs may continue to leak out of treated material.

A review of hazardous substances, undertaken by FRS in 2004, highlighted the lack of information on BFRs in the UK marine environment, particularly in Scotland, or on their biological effects. PBDEs, HBCD and TBBP-A are included on the OSPAR List of Chemicals for Priority Action because of their persistence, potential to bioaccumulate and toxicity. Furthermore these compounds are ubiquitous in the environment and have been found in the marine environment far from known sources. Therefore, there is a need to monitor these compounds in environmental matrices, in Scotland. In addition, PBDEs and HBCD have recently been included on the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP) on a voluntary basis (pre-CEMP). However, it is likely that they will be reclassified as mandatory determinands. Hazardous substances for which it has been agreed that they should be monitored as part of the CEMP but for which guidelines, quality assurance tools and assessment tools are currently unavailable, are included in the pre-CEMP. Their current voluntary status means that only a limited amount of data is being gathered for PBDEs and HBCD across the OSPAR maritime area and it will be this limited dataset which will contribute to assessments of concentrations of these synthetic chemicals in the marine environment. As the ultimate aim of the OSPAR Strategy for Hazardous Substances is to achieve concentrations in the marine environment of near background for naturally occurring [hazardous] substances and close to zero for man-made, synthetic chemicals by 2020, gathering data at an early stage is critical.

An analytical method for the analysis of PBDEs was developed for both sediment and biota at FRS ML as part of the 2006 - 2007 Schedule of Service for the Marine Management Division of the Marine Group, with the aim of undertaking a monitoring programme to establish concentrations in the Scottish marine environment. Following on from this, sediment and biota from a number of locations around Scotland were analysed for PBDEs. Samples were from both remote, and potentially contaminated, areas such as the former sewage sludge dump site at Garroch Head in the Clyde. PBDEs were detected in fish and shellfish from a range of locations across Scotland. Concentrations and profiles were generally in line with
literature values and tissue type. The influence of sewage sludge dumping was apparent in the Clyde. Further analysis is underway with a focus on determining the source of the high BDE209 concentrations and establishing if this is localised to Garroch Head or more widely spread across the Clyde area. Sediment and fish samples are now also being analysed as part of the UK Clean Seas Environment Monitoring Programme (CSEMP). However, at present data on HBCD and TBBP-A is scare, particularly in the Scottish marine environment. As a result of the restriction on the use of the penta and octa-PBDE mixes there has been an increase in the use of other BFRs such as HBCD and TBBP-A. HBCD and TBBP-A are currently among the most widely used BFRs, with an estimated global use of 16,700 and 130,000 tonnes, respectively. As the usage of HBCD and TBBP-A began more recently when compared to PBDEs, and they continue to be produced, it is possible that concentrations in the environment will rise. HBCD has been produced in the UK and although TBBP-A has not, it is imported.

**HEXABROMOCYCLODODECANE (HBCD)**

HBCD was produced in the UK, between 1996 and 2003, at the Newton Aycliffe plant in the north east of England. HBCD is a brominated alicyclic hydrocarbon. It 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 that is predominately used in expanded and extruded polystyrene for thermal insulation foams used in building and construction, and in textile back coatings. HBCD can be released to the environment during its production and while manufacturing other products, and during disposal of products containing this chemical. In addition, HBCD may continue to leak out of 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.

Theoretically, there are sixteen possible stereoisomers of HBCD: 6 diastereomeric pairs of enantiomers and 4 meso forms. However, in technical HBCD mixtures mainly three of the 6 enatiomeric pairs are found, namely α-, β- and γ-HBCD, with the dominant isomer being γ-HBCD. In sediment the γ-isomer also dominates but in biota the major isomer is α-HBCD. β-HBCD is always a minor component. HBCD has a relatively high octanol water partition coefficient (Log K_{ow} = 5.8) and, the potential to bioaccumulate. The hydrophobic nature of HBCD means that it will associate with particulate material and will accumulate in sediment, particularly if it has a high organic carbon content. A BCF of 18,100 l kg⁻¹ has been estimated. 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 characterised and the risks to human health have not been fully evaluated. HBCD has been shown to have sublethal effects in juvenile rainbow trout. Induction of catylase activity and antagonistic effects on CYP1A and an increase in the liver somatic index were observed after 5 days exposure of juvenile trout to HBCD. Developmental neurotoxic effects have also been observed in neonatal mice. Limited endocrine disruption of the reproductive and thyroid hormonal systems were observed in juvenile flounder at HBCD concentrations of up to 446 µg g⁻¹ lipid weight in flounder muscle.

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1Diastereoisomers are stereoisomers that are not mirror images of each other
2A meso compound is one whose molecule is super imposable on its mirror images even though they contain chiral centres
TETRABROMOBISPHENOL-A (TBBP-A)

In terms of its worldwide production, TBBP-A is the largest BFR, however, it has never been produced in the UK. TBBP-A is used primarily in electronics and plastics. It is mainly used as a reactive BFR due to the two hydroxyl groups which allow it to be covalently bound in the polymer matrix. However, it is also used additively (10% of production), which increases the risk of leaching from treated products. The molecular structure of TBBP-A is similar to that of the thyroid hormone, thyroxines, except the iodine atoms have been replaced with bromine atoms. TBBP-A is expected to be present in an ionised form in the marine environment. TBBP-A has a relatively high octanol water coefficient ($\log K_{ow} = 4.5$) and is therefore likely to bioaccumulate. However, the highest measured bioconcentration factor (BCF) is 1,234 l kg$^{-1}$ and is below the OSPAR cut-off point for bioaccumulation of 2,000 l kg$^{-1}$. This is thought to be due to TBBP-A being metabolized by fish. Data for the acute toxicity in fish of TBBP-A has been reported ($LC_{50} = 0.51$ mg l$^{-1}$) and chronic toxicity has been measured with a no-observable-effect concentration (NOEC) of 0.16 mg l$^{-1}$. The $LC_{50}$ value for TBBP-A meets with the DYNAMEC toxicity criterion ($\leq 1$ mg l$^{-1}$).

Limited endocrine disruption of the reproductive and thyroid hormonal systems were observed in juvenile flounder at TBBP-A levels of up to 4300 ng g$^{-1}$ lipid weight in flounder muscle. A study of the toxicological effects of TBBP-A on zebrafish indicated a reduced reproductive success at environmentally relevant concentrations. Neurobehavioural effects were not observed in adult mice exposed to TBBP-A.

ENVIRONMENTAL CONCENTRATIONS OF HEXABROMOCYCLODODECANE (HBCD) AND TETRABROMOBISPHENOL-A (TBBP-A)

Levels and trends of BFRs (including HBCD and TBBP-A) have been reviewed in the European and Arctic environments. Most studies concentrate on the PBDEs and there is limited data on concentrations of HBCD and TBBP-A in the marine environment.

HBCD and TBBP-A were measured in the Scheldt estuary in mysid shrimp and sediment. TBBP-A was only found in trace amounts in mysid and was below the detection limits in sediment. HBCD (sum of $\alpha$-, $\beta$-, $\gamma$-HBCD) was detected in both sediment ($14 – 71$ µg kg$^{-1}$ dry weight) and mysid ($562 - 727$ µg kg$^{-1}$ lipid weight). The environmental occurrence of HBCD was also investigated in Sweden. HBCD was found in a range of matrices (water, soil, sediment, biota) with the highest total concentrations (determined by GC-MS) found in pike ($27$ µg kg$^{-1}$ lipid weight) and in sediment ($25$ µg kg$^{-1}$ dry weight). Morris et al. investigated HBCD and TBBP-A in sediment and biota from North Sea estuaries and rivers using liquid chromatography-mass spectrometry (LC-MS). The highest $\Sigma$HBCD and TBBP-A concentrations in sediment were from the River Skerne, County Durham, close to the Newton Aycliffe plant, with concentrations of $1.7$ mg kg$^{-1}$ dry weight and $9.8$ mg kg$^{-3}$ dry weight, respectively. HBCD had been produced at this plant but not TBBP-A, therefore the high TBBP-A concentrations are unexpected but no explanation is given for this in the paper. Sediment from the River Clyde were also analysed for HBCD and TBBP-A as part of this study. $\Sigma$HBCD concentrations ranged from 7 to 187 µg kg$^{-1}$ dry weight which was lower than found close to the Newton Aycliffe plant but higher than found in other rivers such as the Mersey ($22$ µg kg$^{-1}$ dry weight) and Humber ($6$ µg kg$^{-1}$ dry weight). However, TBBP-A was not detected in the Clyde. The HBCD pattern was similar to the commercial formulations with the $\gamma$-HBCD being the
dominant diastereoisomer. Concentrations were also measured in different trophic levels of the North Sea food web\textsuperscript{16}. Most samples contained α-HBCD, typically the dominant congener in biota. Few samples contained TBBP-A. Concentrations for ΣHBCD ranged from 2.1 to 6.8 mg kg\(^{-1}\) lipid weight in liver and blubber of harbour porpoises and seals with some evidence of biomagnification through the food web. HBCD and TBBP-A were also measured in the blubber of stranded porpoises found in the UK between 1994 and 2004\textsuperscript{17}. TBBP-A concentrations ranged from 6 – 35 µg kg\(^{-1}\) wet weight. α-HBCD was the dominant HBCD isomer with concentrations ranging from 10 to 19,200 µg kg\(^{-1}\) wet weight. Zegers \textit{et al.} investigated HBCD concentrations in harbour porpoises and dolphins from western European Seas\textsuperscript{18}. The highest HBCD concentration was in harbour porpoises stranded on the Irish and Scottish coasts of the Irish Seas (2.9 mg kg\(^{-1}\) lipid weight) and on the north west coast of Scotland (5.1 mg kg\(^{-1}\) lipid weight). Concentrations in other areas were < 1.5 mg kg\(^{-1}\) lipid weight. α-HBCD was the only diastereoisomer detected. HBCD was found in harbour porpoise sampled from Scottish waters between 2001 and 2003\textsuperscript{19}. Concentrations for ΣHBCD ranged from 393.3 to 9,592 µg kg\(^{-1}\) lipid weight (mean = 2,354 µg kg\(^{-1}\) lipid weight) and marine fish (4.9 and 4 µg kg\(^{-1}\) wet weight) from the Arctic and TBBP-A in marine sediment (1.24 µg kg\(^{-1}\) dry weight) and fish (0.35 and 1.31 µg kg\(^{-1}\) wet weight)\textsuperscript{13}. α-HBCD was also found in small cetaceans (dolphins and porpoises) from the South China Sea between 1990 and 2001 and concentrations were found to have increased significantly with mean concentrations in porpoise ranging from 9.5 µg kg\(^{-1}\) lipid weight in 1990 to 35 µg kg\(^{-1}\) lipid weight in 2000/01\textsuperscript{20}. HBCD was measured in mussels from Korea\textsuperscript{21}. Concentrations ranged from 6.0 – 500 µg kg\(^{-1}\) lipid weight with α-HBCD being the dominant isomer in most samples. However, the proportion of γ-HBCD was high compared to other aquatic biota, this was thought to be due to ingested sediment particles. γ-HBCD is the dominant isomer in sediment. An increasing trend in HBCD concentrations was detected in Californian sea lions. Between 1993 and 2003 total HBCD concentrations increased from 0.7 to 12 µg kg\(^{-1}\) wet weight in sea lion blubber\textsuperscript{22}.

HBCD was also measured in farmed salmon as part of an Irish study of BFRs\textsuperscript{23}. Seven fish fillets from seven different sites were analysed for HBCD in addition to PBDEs. HBCD was found in all samples. The mean upperbound concentration for HBCD was 1.17 µg kg\(^{-1}\) wet weight.

An analytical method for the analysis of HBCD and TBBP-A was developed and validated for biota at FRS ML as part of the 2007 - 2008 Schedule of Service for the Marine Management Division, with the aim of undertaking a monitoring programme to establish concentrations in the Scottish marine environment.

**METHOD DEVELOPMENT AND VALIDATION**

**Extraction and Clean-Up Methods for HBCD and TBBP-A**

HBCD and TBBP-A are lipophilic and, therefore, can potentially concentrate in the lipids of an organism. Tissue extracts will always contain many compounds other than HBCD and TBBP-A, and a suitable clean up is necessary to remove those compounds which may interfere with the subsequent analysis. HBCD can be extracted using extraction techniques used for other lipophilic, non-polar compounds such as CBs and PBDEs\textsuperscript{24}. A range of extraction methods have been used for the extraction of HBCD and TBBP-A from biota and sediment. Extraction methods for environmental samples include the more traditional methods such as Soxhlet or Ultra
Turax homogenisation and newer automated methods such as pressurised liquid extraction (PLE). However, most laboratories are still using the traditional Soxhlet extraction. For Soxhlets, hexane/acetone mixtures are commonly used combined with an extraction time of between 6 and 24 hrs. 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 are therefore the preferred methods with PLE having the advantage of using less solvent, being fully automated and taking less time than Soxhlet. Marvin et al. used PLE for the extraction of HBCD from sediment.25 Ashizuka et al. used a PLE method for the extraction of PBDEs in marine products.26 Hexane was used for the extraction, followed by a separate clean-up involving a sulphuric acid wash and silica and florisil columns. Recoveries of between 57.7 and 78.5% were achieved. Samara et al. also used PLE for the extraction of PBDEs from sediment, followed by an alumina column clean up using hexane:DCM (1:2, v/v).27

One advantage of using PLE extraction is that it is possible to combine the clean up with the extraction, especially where mass spectrometry is being used as the detection method. Methods have been developed by Lund University for online clean-up and fractionation of dioxins, furans and PCBs with PLE for food, feed and environmental samples.28 The first method utilises a fat retainer for the on-line clean-up of fat. Silica impregnated with sulphuric acid, alumina and florisil have all been used as fat retainers. A non-polar extraction solvent such as hexane should be used if fat retainers are used during PLE.

A PLE method was developed at FRS for the extraction of PBDEs in both sediment and biota. The development and validation of this method is described in a previous report. In summary, sediment and biota were extracted by PLE using iso-hexane. Addition of 5% deactivated alumina (biota, 30 g; sediment 15 g) to the PLE extraction tubes allowed the extraction and clean-up steps to be combined. The extract was then concentrated using a Turbovap prior to analysis by GC-ECNIMS.

The PBDE PLE method, using fat retainers, was also investigated at FRS ML for the extraction of HBCD and TBBP-A (Fig. 1). Initially no matrix was used and the PLE cell was filled with sodium sulphate and alumina (5% deactivated) and spiked with HBCD and TBBP-A. Good recoveries (>85%) of HBCD were obtained using 5g of alumina. However, this was not sufficient to clean up biota samples for HBCD analysis as not all lipid material could be removed. Addition of more alumina resulted in losses of HBCD. If a higher alumina deactivation level was used or more polar solvents then lipid was found to breakthrough. A non-polar solvent must be used to avoid lipid breakthrough, therefore, iso-hexane is used for the extraction. Therefore, a second clean up step was required. TBBP-A was retained in the alumina in the PLE cell using 5g of 5% deactivated alumina. Therefore, fat retainer could not be used for TBBP-A and an alternative clean-up was required.

The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography, but gel permeation chromatography (GPC) can also be employed. Column chromatography, using alumina, silica or florisil, was investigated as a possible second clean-up step. However, TBBP-A could not be easily separated from the lipid. More polar solvents were required to elute TBBP-A but this would also result in lipid breakthrough. Therefore, GPC was investigated. Two serial columns were used as this improves lipid separation. Dichloromethane/iso-hexane (1:1; v/v) was used as the mobile phase at a flow rate of 5 ml min⁻¹. The majority of co-extractives were eluted from the GPC columns in the first 160 ml. TBBP-A was eluted in 160 - 240 ml and the three HBCD diastereoisomers 170 – 320 ml (Fig. 2). The exact elution volume was pre-determined by injecting the stock solution, collecting fractions and analysing by GC-ECD. To minimise co-extractives that may
interfere with the LC-MS analysis, the 170 – 320 ml fraction was collected from the GPC; the use of labelled standards will correct for the loss of TBBP-A in the 160 – 170 ml (~ 5%). The solvent was removed by rotary evaporation and the residue reconstituted in methanol (0.5 ml) for analysis by LC-MS. If no fat retainer was used and GPC only was used as the clean up, there were problems quantifying HBCD by LC-MS due to co-elution in the chromatogram. TBBP-A, which eluted first from the HPLC column, was well separated from any interferences and there were no co-elution problems. Therefore, for HBCD analysis it was necessary to use 5 g of fat retainer in the PLE cell followed by GPC and for TBBP-A GPC alone was sufficient. A combined method for HBCD and TBBP-A was therefore not possible and HBCD and TBBP-A had to be extracted separately.

Instrumental Determination of HBCD and TBBP-A

Developments in the analysis of BFRs, including HBCD and TBBP-A, in environmental samples were recently reviewed. Analysis of HBCD is less straightforward than the analyses of PBDEs and a different approach is normally 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. In addition, the three main HBCD diastereoisomers found in technical mixtures cannot be separated by GC and a total concentration only can be determined. A liquid chromatography (LC) method is required to separate the three diastereoisomers, with separation of enantiomers being possible with a chiral HPLC column.

GC-MS

Few publications analyse HBCD along with the PBDEs by GC-MS, although it has been done using both GC-electron capture negative ionisation (ECNI) and high resolution GC-MS. GC-electron capture detection (ECD) is rarely used due to the limited linear range, and lack of selectivity. If GC-ECD is used then the clean-up will need to separate out all other organohalogenated compounds which may give co-elution problems. However, a GC-ECD method was set up at FRS for the determination of the GPC elution volume only. This detection method was adequate for the analysis of HBCD and TBBP-A solutions eluting from the GPC column, as there was no sample matrix and therefore no co-elution problems.

Both high and low resolution GC-MS can be used in conjunction with either electron ionisation (EI) or ECNI. Most laboratories using GC for HBCD used low resolution GC-MS, normally in ECNI mode. ECNI shows improved sensitivity compared to EI or positive impact chemical ionisation (PCI). When GC-ECNIIMS is used, the bromine ion is monitored. One of the drawbacks of the CI mode is that isotopically labelled standards (13C) cannot be used as internal standards for quantification purposes when only the bromine ions are monitored. Larger fragment ions, required for structural confirmation are not formed in ECNI mode. One of the main issues with using GC-MS for the analysis of HBCD is that the HBCD isomers interconvert at temperatures >160°C, therefore the three HBCD diastereoisomers (α-, β-, γ-HBCD) cannot be separated and a broad hump is obtained in the GC chromatogram. In addition, the three diastereoisomers will have different response factors and, therefore, the concentration of HBCD cannot be determined accurately by GC-MS. Furthermore HBCD degrades at 240°C, therefore, there may be significant losses of HBCD during GC analysis. Cold on-column injection, short GC columns and thin stationary films can minimise 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. Co-elution of HBCD with certain PBDEs can also be a problem.
Verslycke et al. analysed for TBBP-A and HBCD along with the PBDEs using GC-ECNIMS, but gave no details of the method or validation data. Oberg et al. analysed PBDEs and HBCD using high resolution GC-MS but again no information on the method performance was given.

Initially attempts were made at FRS to analyse for HBCD and TBBP-A using the GC-ECNIMS method for PBDEs. However, reproducibility and sensitivity were poor. HBCD gave a broad unresolved peak due to the three diastereoisomers. Both compounds were separated from the PBDEs, however, HBCD and TBBP-A were not always recovered and were generally only detected at higher concentrations (standard solutions of concentrations > 20 ng ml⁻¹ for TBBP-A and > 5 ng ml⁻¹ for total HBCD). Even above these concentrations it was not always possible to detect HBCD and TBBP-A as both compounds can breakdown during the GC separation.

**LC-MS**

LC-MS is the preferred technique for both HBCD and TBBP-A and eliminates the need for a derivatisation step for TBBP-A. Morris et al. determined TBBP-A and HBCD in sediments and biota using LC-MS. LC-MS has also been used to determine TBBP-A is sediment and sewage sludge.

Due to the problem with GC-MS for the analysis of HBCD and TBBP-A an LC-MS method was developed at FRS for the analysis of HBCD and TBBP-A. A reverse phase column (C₁₈) was used for analysis of HBCD and TBBP-A by LC-MS. The three diastereoisomers (α-, β-, γ-HBCD) found in the technical mixture were separated using acetonitrile/water, with ammonium acetate (10 mM), as the mobile phase (Fig. 3a). The flow rate was 250 µl min⁻¹ and a gradient programme was required (Table 1). HPLC with chiral columns such as permethylated β-cyclodextrin columns can also be used to separate the enantiomers of the α, β, γ-HBCD diastereoisomers.

Electrospray ionisation was used. The optimised parameters used for HBCD and TBBP-A are shown in Table 2. Electrospray is more sensitive than atmospheric pressure chemical ionisation (APCI) and is therefore the most frequently used technique for LC-MS analysis. The deprotonated molecular ion (m/z = 641.0) was the major ion for all HBCD isomers and for TBBP-A (m/z = 543.1). There were no major fragment ions identified to be used as qualifier ions. Ion suppression and matrix effects are a common problem with LC-MS analysis. This can be alleviated by having an efficient clean-up procedure and through the use of labelled internal standards. Deuterated and ¹³C-labelled HBCD standards are available for the three major diastereoisomers of HBCD and ¹³C–TBBP-A is also available. However, deuterated standards are less expensive and are therefore the preferred option for HBCD. Therefore, deuterated α, β, γ-HBCD (m/z = 658.2) and ¹³C-TBBP-A (m/z = 554.8) were used as internal standards LC-MS has been reported to have poorer detection limits compared to GC-MS, with the sensitivity being approximately 10 times less than that of the GC-NCIMS method. Using LC-MS and with an injection volume of ~15 µl, it should be possible to detect around 0.5 ng on column. LC-MS-MS can usually overcome the problem of higher detection limits.

**Method Validation for HBCD and TBBP-A**

For the validation of the method the following was investigated:

- linear response range
- precision of standards and samples
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- limit of detection
- recovery
- blank values

Linear Response Range

The linear response range was assessed by the triplicate analysis of seven standards ranging from 10 ng ml\(^{-1}\) to 1000 ng ml\(^{-1}\) for HBCD and TBBP-A. The injection volume was 15 \(\mu\)l. Quadratic calibration curves gave correlation coefficients of at least 0.996. Non linear curves occur frequently in LC-ESI-MS. The decrease in response at high concentrations is often attributed to analyte saturation. Morris et al. reported a limited linear range (20 – 250 ng µl\(^{-1}\)) for the analysis of HBCD using LC-ESI-MS and used a second order curve for sample quantification\(^{32}\).

Precision of Standards and Samples

For the determination of analytical precision, replicate low (100 ng ml\(^{-1}\)) and high (900 ng ml\(^{-1}\)) standards of the working range were analysed on separate days. Table 3 shows the precision for the BFRs at both concentrations. CV\% for the HBCD and TBBP-A were <10\% for both the high standard and low standard. Precision of the whole method was calculated through the replicate analysis of a spiked sample on separate days. CV\% were again <10 \% for HBCD and TBBP-A.

Limits of Detection

The limits of detections (LoD) of HBCD isomers and TBBP-A were determined through the repeat analysis (n =7) of a low spiked sample consecutively on the same day. Biota (Cod liver oil, 0.5 g) were spiked with 15 ng of each compound, and labelled standard, and left overnight before extraction. Following PLE extraction (including alumina as fat retainer for HBCD) and clean up by GPC and concentration to ~ 0.5 ml, samples were analysed by LC-MS in one batch. The mean concentration and standard deviation for each congener were calculated and the LoDs calculated from 4.65 x S.D. (Table 3). The LoDs for biota samples were around 0.3 \(\mu\)g kg\(^{-1}\) wet weight for the HBCD and TBBP-A for the extraction of a 10 g sample and around 6 \(\mu\)g kg\(^{-1}\) wet weight for a 0.5 g sample (Table 3). Few publications give method LoDs. Morris et al. report limits of quantification of 1.2 \(\mu\)g kg\(^{-1}\) (biota and sediment) based on the extraction of 1 g and an injection volume of 15 \(\mu\)l for analysis by LC-ESI-MS\(^{32}\). This is around half of the FRS LoD for a similar sample size.

Recovery

Recoveries were calculated through replicate analysis of HBCD and TBBP-A in cod liver oil (0.5 g) samples on separate days. The spiking solution was added to the sample and sodium sulphate mixture prior to drying overnight. Cod liver oil was spiked with 100 ng (TBBP-A) and 50 ng (HBCD) of each diastereoisomer. The samples were then extracted by PLE (using fat retainer for HBCD), cleaned-up by GPC, concentrated by Syncore and analysed by LC-MS. Recoveries are shown in Table 4 and typical chromatograms in Figure 4. Average recoveries for \(\alpha\)-, \(\beta\)- and \(\gamma\)-HBCD were 105.3\% (CV\% = 11.3, n = 8), 107.5\% (CV\% = 7.8, n = 8) and 86.3\% (CV\% = 12.3, n = 8), respectively, and for TBBP-A 101.3\% (CV\% = 3.5\%, n = 8). Morris et al. reported recoveries of 127\% (CV\% = 27\%), 101\% (CV\% = 11\%) and 80\% (CV\% = 9\%) for \(\alpha\)- \(\beta\)- and \(\gamma\)-HBCD, respectively\(^{32}\).
Procedural Blanks

With each batch of samples a procedural blank was analysed. The matrix/sodium sulphate mixture (Fig. 1) was replaced with sodium sulphate and extracted by PLE (with alumina for HBCD), cleaned-up by GPC, concentrated by Syncore and analysed by LC-MS. Concentrations of the three HBCD diastereoisomers and TBBP-A compounds were below the LoDs.

ANALYTICAL METHODOLOGY

Cleaning of Glassware, PLE Tubes, Filters and Sodium Sulphate

At FRS ML glassware was washed and dried in a GW 4000 glassware washer (Camlab Ltd., Cambridge, UK). Prior to use, all glassware was rinsed twice with acetone and then twice with iso-hexane, the latter being allowed to evaporate before proceeding. Anhydrous sodium sulphate was washed ultrasonically with dichloromethane (DCM) (2 x 500 ml) followed by iso-hexane (2 x 500 ml) and dried overnight at 150°C.

Glass fibre filters were wrapped in aluminium foil and placed in a muffle furnace set to 200°C for 12 hours. All pressurised liquid extraction (PLE) cells, caps and collection bottles were solvent washed with acetone followed by iso-hexane, with the latter being allowed to evaporate before proceeding. The lids of the collection bottles were fitted with ultra low bleed septa which were first solvent washed with iso-hexane.

Lipid Determination

The total lipid content was determined using the Smedes method. The biota sample (0.5 – 10 g) was weighed into a centrifuge tube and iso-propanol (18 ml) and cyclohexane (20 ml) added. The sample was homogenised then de-ionised water (~13 – 22 ml, depending on the moisture content of the sample) added and the mixture homogenised again. Centrifugation was used to separate the organic extract from the particulate material. A second extraction was carried out with 13% (w/w) iso-propanol in cyclohexane. The two extracts were combined, concentrated by rotary evaporation and dried in a oven for at least 1 hour at 150°C ± 5°C. The residue was weighed and the lipid content calculated.

Preparation of Standard Solutions

Standards were obtained as single compound solutions at a concentration of 50 µg ml⁻¹ from LGC Promochem, (Middlesex, UK). These were HBCD (α, β, γ isomers) and TBBP-A. Calibration standards were prepared by mixing each of the solutions and making up to 10 ml in iso-hexane to give a 1 µg ml⁻¹ composite stock solution. Dilutions were made to give six calibration standards with nominal concentrations of 500, 250, 100, 10, 5 and 1 ng ml⁻¹. Labelled internal standards, at a concentration of 100 ng ml⁻¹, were also included in the calibration standards. All calibration standards were analysed by liquid chromatography-mass spectrometry (LC-MS).
Preparation for Pressurised Liquid Extraction (PLE)\(^{iii}\)

An appropriate amount of tissue (equivalent to 300 mg lipid) was mixed with sodium sulphate (~20 g), labelled internal standards added (\(^{3}\)H-\(\alpha\)-, \(\beta\)- and \(\gamma\)-HBCD and \(^{13}\)C-TBBP-A), and left overnight before being ground to a fine powder using a mortar and pestle. Solvent washed PLE cells (100 ml) were packed as follows: solvent washed filter paper, pre-washed sodium sulphate (10 g), 5% deactivated alumina (5 g, HBCD only), solvent washed filter paper and the biota/sodium sulphate mixture prepared as above. The cell was finally filled to the top with more sodium sulphate then packed down and topped up if required and another filter paper placed on top (Fig. 1). It was essential that the cell was tightly packed, so as to minimise the dead volume.

Pressurised Liquid Extraction (PLE)

Samples were extracted using an oven temperature of 60°C and a pressure of 1500 psi. Five minutes heating was followed by 2 x 5 min static cycles. The cell flush was 50% total cell volume (\(i.e.\) 25 % of the cell volume for each flush = 25 ml per flush) with a 60 second purge (using nitrogen) at end of each sample extraction. The extraction solvent used was iso-hexane. Following PLE extraction, the extracts were transferred to Syncore tubes and the volume reduced to ~0.3 ml.

Gel Permeation Chromatography (GPC)

Any remaining lipid and other co-extractives were separated from HBCD and TBBP-A using gel permeation chromatography (GPC). A guard column and Phenomenex phenogel columns (300 x 21.2 mm, 50 Å) were connected in series. The mobile phase used was dichloromethane / iso-hexane (1:1, v/v) with a flow of 5 ml min\(^{-1}\). An aliquot of the iso-hexane extract was injected onto the column via a rheodyne valve. The first fraction collected (170 ml) was discarded the second fraction (170 - 320 ml) containing the HBCD diastereoisomers and TBBP-A was collected and the solvent removed using a rotary evaporator. The exact elution volume was pre-determined by injecting 1 ml of the stock standard (1 µg ml\(^{-1}\)) and collecting fractions for analysis of HBCD (sum of diastereoisomers) and TBBP-A by GC-electron capture detection (ECD). The cleaned-up extract was reconstituted in methanol, mixed using a whirlimixer and analysed by LC-MS using a reverse phase C\(_{18}\) column.

Analysis by Liquid Chromatography-Mass Spectrometry (LC-MS)

A PE Sciex API 150 (Perkin Elmer, Maclesfield, UK) single quadropole mass spectrometer equipped with an electrospray source was utilised for the analysis. The LC mobile phase used was acetonitrile/ water, using ammonium acetate as a modifier. The flow rate was set at 200 µl min\(^{-1}\) using an HP1100 quaternary pump, the gradient used is shown in Table 1. The run time was 45 minutes. A 150 x 2.00 mm ID column packed with 3 \(\mu\)m particles coated with a C\(_{18}\) stationary phase was used.

The tuning parameters were optimised in the positive ion mode by injecting 10 µl of a 1 µg ml\(^{-1}\) stock solution directly onto the mass spectrometer. The optimum parameters are shown in Table 2. A method was also set up for quantification purposes in single ion monitoring (SIM) mode. A six-point calibration curve was used for quantification. The ions monitored were \(m/z\) 641.0 (HBCD), 658.2 (\(^{3}\)H-HBCD), 543.1 (TBBP-A) and 554.8 (\(^{13}\)C-TBBP-A) with a dwell time of 150 msecs.

\(^{iii}\) Formerly referred to as accelerated solvent extraction (ASE)
The method and standard operating procedure (SOP) for the analysis of HBCD and TBBP-A are shown in Appendix 1.

CONCLUSIONS

1. Brominated flame retardants (BFRs) have a high usage and can undergo long-range atmospheric transport. Data is now available for PBDEs in the Scottish aquatic environment, however, there is limited data on other BFRs such as HBCD and TBBP-A. However, it is likely that they will be found, both in biota and sediments from Scottish waters.

2. A review of analytical methods used for the analysis of HBCD and TBBP-A was undertaken. LC-MS is the most commonly used technique for this analysis and has the advantage over GC-MS in that HBCD diastereoisomers can be separated.

3. An LC-MS method was developed for HBCD and TBBP-A. The three HBCD diasteriosomers were separated using a C18 column and acetonitrile/water mobile phase. Labelled internal standards were used to improve quantification and reduce any matrix effects.

4. A range of extraction methods have been used including traditional Soxhlet and newer automated methods such as pressurised liquid extraction (PLE). Most publications employed a separate clean up using gel permeation, alumina or silica column or sulphuric acid treatment.

5. One advantage of using PLE is that the extraction and clean-up may be combined by adding fat retainers to the extraction cell. This technique is currently used at FRS for the extraction of PBDEs from sediment and biota.

6. The extraction and clean-up method developed at FRS for PBDEs was investigated for HBCD and TBBP-A. For PBDEs using 5% deactivated alumina in the PLE cell it was possible to extract and clean-up sediment and biota (= 300 mg lipid) samples. However, using this method, TBBP-A was lost and recoveries of HBCD with 30 g fat retainer were poor, although acceptable with 5 g fat retainer. Therefore, an alternative clean up step was required.

7. A gel permeation chromatography (GPC) method was developed for the clean-up of biota samples for HBCD and TBBP-A analysis. PLE followed by GPC was sufficient for TBBP-A analysis by LC-MS. However, if this method was used for HBCD there was coelution in the LC-MS chromatogram. Using the fat retainer in the PLE prevented this. Therefore, a combined method for HBCD and TBBP-A in biota was not possible and methods for HBCD and TBBP-A were validated separately.

8. Replicate analysis of high and low standards on separate days by LC-MS gave CV% of <10% for HBCD and TBBP-A.

9. Recoveries for HBCD and TBBP-A from biota were mainly > 95%.
10. The LoDs for biota samples were dependent on the sample size. For small sample sizes (e.g., fish liver- 0.5 g) the LoD was around 6 μg kg\(^{-1}\) wet weight and for larger samples (e.g., fish flesh or mussels- 10 g) an LoD of 0.3 μg kg\(^{-1}\) wet weight was achievable.

REFERENCES


4. L. Webster, M. Russell, P. Walsham and C. F. Moffat, 2006, A review of brominated flame retardants (BFRs) in the aquatic environment and development of an analytical technique for their analysis in environmental samples, Fisheries Research Services Internal Report No 06/06.


TABLE 1

Gradient LC mobile phase programme for the separation of TBBP-A and the HBCD diastereoisomers.

<table>
<thead>
<tr>
<th>Total time (min)</th>
<th>Flow rate (µl/min)</th>
<th>A %</th>
<th>B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>10.0</td>
<td>250</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>32.0</td>
<td>250</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>40.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>45.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

A, water with ammonium acetate (10 mM)
B, acetonitrile with ammonium acetate (10 mM)

TABLE 2

Optimised parameters for the analysis of TBBP-A and the HBCD diastereoisomers by LC-MS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebuliser Gas (N₂)</td>
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</tr>
<tr>
<td>Curtain Gas (N₂)</td>
<td>10</td>
</tr>
<tr>
<td>Nebuliser Temperature</td>
<td>350°C</td>
</tr>
<tr>
<td>Ion Spray voltage</td>
<td>-2000</td>
</tr>
</tbody>
</table>
TABLE 3

Precision of low (100 ng ml⁻¹; n = 7) and high (900 ng ml⁻¹; n = 9) standards and method LoD determined from the replicate (biota n = 7) analysis of a spiked sample at a low concentration (4.65 x SD).

<table>
<thead>
<tr>
<th></th>
<th>90% standard %CV</th>
<th>10% standard %CV</th>
<th>Instrument LoD (ng ml⁻¹)</th>
<th>LoD biota (fish liver ~0.5 g) (μg kg⁻¹ wet weight)</th>
<th>LoD biota (fish muscle or mussels ~10 g) (μg kg⁻¹ wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HBCD</td>
<td>7.2</td>
<td>5.2</td>
<td>2.97</td>
<td>6.9</td>
<td>0.35</td>
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<tr>
<td>β-HBCD</td>
<td>5.3</td>
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<tr>
<td>γ-HBCD</td>
<td>9.9</td>
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<td>2.28</td>
<td>7.9</td>
<td>0.39</td>
</tr>
<tr>
<td>TBBP-A</td>
<td>7.5</td>
<td>9.4</td>
<td>1.12</td>
<td>6.9</td>
<td>0.35</td>
</tr>
</tbody>
</table>

TABLE 4

Summary of results from spiking experiments. Mussels were spiked with HBCD (n = 8) and TBBP-A (n = 6) and the recovery and CV% calculated for the three HBCD diastereoisomers and TBBP-A.

<table>
<thead>
<tr>
<th></th>
<th>% recovery</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HBCD</td>
<td>105.3</td>
<td>11.4</td>
</tr>
<tr>
<td>β-HBCD</td>
<td>107.5</td>
<td>7.8</td>
</tr>
<tr>
<td>γ-HBCD</td>
<td>86.3</td>
<td>12.3</td>
</tr>
<tr>
<td>TBBP-A</td>
<td>101.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Figure 1 Packing of pressurised liquid extraction (PLE) tubes for the extraction of HBCD and TBBP-A from biota. For TBBP-A no fat retainer was used.
Figure 2 Elution of HBCD, TBBP-A and lipid from gel permeation chromatography (GPC) columns.
Figure 3 Single ions chromatogram of a standard (100 ng ml\(^{-1}\)) containing (a) α-, β- and γ-HBCD \((m/z = 641.0)\) and (b) TBBP-A \((m/z = 543.1)\), analysed by LC-MS.
Figure 4: Single ions chromatogram of a spiked sample (a) α-, β- and γ-HBCD ($m/z = 641.0$; 50 ng) and (b) TBBP-A ($m/z = 540.9$; 100 ng), analysed by LC-MS. For TBBP-A alumina was not used as a fat retainer in the PLE cell.
APPENDIX 1

Methods and Standard Operating Procedure (SOP) for the analysis of HBCD and TBBP-A in Biota
Determination of Hexabromocyclododecane and Tetrabromobisphenol-A in Biota by LC-MS- M0660

1. Introduction and Scope

This method describes the determination of hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBP-A) in biota. The analysis incorporates three of the diastereoisomers of HBCD; α-, β- and γ-HBCD. The concentration range of the method is from the limit of detection to 400 ng g⁻¹ (wet weight) for biota.

2. Principle of the Method

HBCD and TBBP-A are extracted from biota by pressurised liquid extraction (PLE). Fat and other co-extractives are removed by PLE with fat retainer (HBCD only) and gel permeation chromatography (GPC). The cleaned up extract is taken to dryness using a rotary evaporator and reconstituted in methanol prior to analysis.

Quantitative analysis is carried out by liquid chromatography with mass spectrometry (LC-MS) using a reverse phase C₁₈ column. Quantification is carried out using an internal standard method using labelled standards; α-, β- and γ-HBCD (d₁₈), and ¹³C TBBP-A.

3. Reference Materials

Spiked Cod Liver Oil

4. Reagents

See SOPs for reagents used.

5. Equipment

LC-MS with autosampler (EN numbers 694 & 695) C₁₈ column
PC (with Analyst 1.1 software) and printer (EN number 1042 & 1043)
Dionex Accelerated Solvent Extractor (ASE 300; EN 1241)
HPLC fitted with a Plgel 10um guard column & 2 Plgel 10um 50A, 300x25mm GPC columns
Syncore evaporator
Rotary Evaporator

6. Environmental Control

See individual SOPs.

7. Interferences
All glassware is solvent washed with acetone followed by iso-hexane. All new batches of iso-hexane and are checked for contamination as outlined in SOP 1620 and analysed by gas chromatography with flame ionisation detection (GC-FID), as described in SOP1610.

8. Sampling and Sample Preparation

Samples are logged into the laboratory according to SOP 0060. Samples are sub-sampled and mixed according to SOP 0605 (section 8).

9. Analytical Procedure

9.1 A procedural blank and a reference material are analysed with each batch of samples.

9.2 The extraction of HBCD and TBBP-A is carried out pressurised liquid extraction (PLE) using a Dionex Accelerated Solvent Extractor (ASE) as detailed in SOP 0605.

9.3 The prepared extracts are cleaned up as outlined in SOP 0610.

9.4 Solvent is removed from the cleaned up extract by rotary evaporation (SOP 1640) and transferred to a HPLC vial prior to analysis.

9.5 Analysis is performed by LC-MS as outlined in SOPs 1715 and 1720.

9.6 Calibration standards required for quantitative analysis, are prepared as described in SOP 0600

10. Calculation of results

The LC-MS is calibrated and results calculated using the Analyst 1.1 software as described in SOP 1710 using an internal standard method. Procedural blanks are evaluated. The concentration is calculated in ng g\textsuperscript{-1} dry weight for sediment and ng g\textsuperscript{-1} wet weight for biota.

Note: if the recovery of the spiked sediment is < 80% the batch must be repeated.

11. Precision, accuracy and practical detection limits

Recoveries were calculated by spiking biota (fish liver and muscle) samples before extraction. Limits of detection are calculated by multiplying the standard deviation of the mean of a low spiked sample by 4.65.

12. Reports

A hardcopy of all data should be obtained and submitted to the Technical Manager along with other relevant documentation (SOP 1350). Batches of results are electronically archived (SOP 030) to duplicate CDs via an internal CD writer. CDs are labelled with archive dates, files checked, group name and contents, one is given to Quality Management for archive and the other is stored in rm C125. Paper copies of chromatograms are kept for one year.
13. **Safety**

Safety for all relevant procedures are provided in the appropriate SOPs detailed above, with reference to risk assessments.

14. **Literature references**

See SOPs 0605, 0371, 0610 & 0600.
15. Performance data – Batch 3470

15.1 Instrument Precision – Low Standard (10% ug ml\(^{-1}\) standard)

<table>
<thead>
<tr>
<th>Date</th>
<th>Analyte</th>
<th>TBBP-A</th>
<th>(\alpha)-HBCD</th>
<th>(\beta)-HBCD</th>
<th>(\gamma)-HBCD</th>
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</thead>
<tbody>
<tr>
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<td>TBBP-A</td>
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<td>105</td>
<td>120</td>
<td>107</td>
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<td>27/06/2006</td>
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<td>99.5</td>
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<td>TBBP-A</td>
<td>93.9</td>
<td>96.9</td>
<td>105</td>
<td>100</td>
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</tbody>
</table>

|          | Mean    | 103.4  | 101.0          | 103.6          | 101.3          |
|          | SD      | 9.7    | 5.3            | 8.9            | 3.4            |
|          | CV%     | 9.4    | 5.2            | 8.5            | 3.4            |

15.2 Instrument Precision – High standard (90% 1 ug ml\(^{-1}\) standard)

<table>
<thead>
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<th>Date</th>
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<tr>
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<td>842</td>
<td>748</td>
<td>798</td>
<td>753</td>
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</tbody>
</table>

|          | Mean    | 929.9  | 851.9          | 880.1          | 891.1          |
|          | SD      | 69.8   | 61.1           | 46.5           | 88.2           |
|          | CV%     | 7.5    | 7.2            | 5.3            | 9.9            |

15.3 Instrument LOD

<table>
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<tr>
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<th>SD</th>
<th>CV%</th>
<th>LOD</th>
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\[ \text{LOD} = 4.65 \times \text{SD} \]
## 15.4 Biota LoD

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<tr>
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<th>Number</th>
<th>LC MS File</th>
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<td></td>
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<td><strong>CV%</strong></td>
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<tr>
<td>LoD (μg kg⁻¹)</td>
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<td>7.60</td>
</tr>
</tbody>
</table>

LoD = (4.65*SD)/ sample weight

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<th>LC MS File</th>
<th>ng in sample TBBP A</th>
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<td>2669/07 15</td>
<td>131107#</td>
<td>16.3</td>
</tr>
<tr>
<td>Cod Liver oil</td>
<td>2669/07 16</td>
<td></td>
<td>15.2</td>
</tr>
<tr>
<td>Cod Liver oil</td>
<td>2669/07 17</td>
<td>141107#13</td>
<td>14.1</td>
</tr>
<tr>
<td>Cod Liver oil</td>
<td>2669/07 18</td>
<td>141107#14</td>
<td>15.8</td>
</tr>
<tr>
<td>Cod Liver oil</td>
<td>2669/07 19</td>
<td>141107#15</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Mean 15.18  
SD 0.74  
CV% 4.90  
LoD (μg kg⁻¹) 6.92

Notebook 169 page 163  
LoD = (4.65*SD)/ sample weight

15.5 Recovery from Spiked sample

<table>
<thead>
<tr>
<th>50 ng spike</th>
<th>Mean</th>
<th>Mean % Recovery</th>
<th>Stddev</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HBCD</td>
<td>50.7</td>
<td>101.4</td>
<td>6.0</td>
<td>11.8</td>
</tr>
<tr>
<td>β-HBCD</td>
<td>53.5</td>
<td>106.9</td>
<td>4.2</td>
<td>7.8</td>
</tr>
<tr>
<td>γ-HBCD</td>
<td>48.1</td>
<td>96.1</td>
<td>10.0</td>
<td>20.8</td>
</tr>
</tbody>
</table>

100 ng spike

<table>
<thead>
<tr>
<th>TBBP-A</th>
<th>Mean</th>
<th>Mean % Recovery</th>
<th>Stddev</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>101.3</td>
<td>101.3</td>
<td>3.9</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Notebook 169 page 151 and 163

16 Uncertainty of Measurement

Sampling:
Sampling not part of method. Samples are analysed and results reported on the samples as received – outwith uncertainty calculations.
Subsampling:
Processing – Error due to inhomogeneity of sample is minimised by mixing thoroughly in sample container - negligible contribution to uncertainty. Injection on Rheodyne – Assume sample in vial is homogenous - negligible contribution to uncertainty. Injection on LC-MS – Assume sample in vial is homogenous – negligible contribution to uncertainty.

Storage:
Samples are stored deep frozen to minimise degradation.

Reagent purity:
All solvents are from Rathburn Chemicals and of at least HPLC Grade, considered sufficient – uncertainty accounted for in validation data. Other chemicals are at least Analar quality, considered sufficient – uncertainty accounted for in validation data. Chemical standards used in the preparation of calibration solutions are of the highest purity available at time of purchase. Final concentrations of the calibration solutions have not been corrected for purity- uncertainty accounted for in the validation data.

Instrument effects:
All syringes are solvent washed between samples. Weight – Tolerance of balance – balances check weight tolerances 0.05% and 0.002%, 2, 3 and 4 decimal places used, sufficient for accuracy required. Uncertainty accounted for in validation data. Volume – Pipettes and syringes used for calibration standards calibrated to <1%. Uncertainty accounted for in validation data. Temperature – Thermometer to measure rotary evaporator water bath temperature calibrated to <1°C. Uncertainty accounted for in validation data. Timer – Timer for HPLC flow calibrated to < 2 sec. Uncertainty accounted for in validation data.

Environmental conditions:
Contamination is minimised by the use of dedicated accommodation, equipment and glassware for organic analysis. Glassware is also separated during cleaning – uncertainty accounted for in validation data.

Computational Effects:
Integration of peaks by means of instrument software. Concentrations calculated by means of internal standard calibration using instrument integrations. Manual checks of peak integrations are made for each sample, negligible contribution.

Blank Correction:
A procedural blank is analysed with each batch of samples. No contribution to uncertainty.

Operator Effects: Only trained personnel may perform method unsupervised. Variations between operators are accounted for by control chart data. Uncertainty accounted for in validation data.

Random Effects: These will be accounted for by validation data.
**Combined uncertainty:**
Systematic component: % recovery of spike = Y.
\[ Y - 100 = \frac{Z}{2} \% = C_s \]

Spike added to all biota is

Random component (CV% Shewhart chart) = \( C_r \)
(random component of LC-MS is from CV% of precision of biota samples as no Shewhart chart data available)

Assume linear summation and a value of K=2:

Combined standard uncertainty = \( (C_s^2 + C_r^2)^{0.5} \) ng

Expanded uncertainty = \( 2(C_s^2 + C_r^2)^{0.5} \) ng

The reported expanded uncertainty is based on an uncertainty multiplied by a coverage factor of K=2, providing a level of confidence of approximately 95%.

<table>
<thead>
<tr>
<th>Determinand</th>
<th>Systematic Component %</th>
<th>Random Component %</th>
<th>Combined Uncertainty %</th>
<th>Expanded Uncertainty %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBP A</td>
<td>0.650</td>
<td>3.5</td>
<td>3.56</td>
<td>7.12</td>
</tr>
<tr>
<td>α HBCD</td>
<td>0.683</td>
<td>11.4</td>
<td>11.42</td>
<td>22.84</td>
</tr>
<tr>
<td>β-HBCD</td>
<td>3.450</td>
<td>7.8</td>
<td>8.53</td>
<td>17.05</td>
</tr>
<tr>
<td>γ-HBCD</td>
<td>-1.950</td>
<td>12.3</td>
<td>12.45</td>
<td>24.9</td>
</tr>
</tbody>
</table>
Accelerated Solvent Extraction of Biota for the determination of HBCD and TBBP-A

1. Introduction and Scope

Weighed samples of biota are placed into extraction cells and extracted with iso-hexane HBCD and TBBP-A. If HBCD is to be analysed, partial lipid removal from samples is achieved by the addition of 5% deactivated alumina to the extraction cells with final lipid removal by Gel Permeation Chromatography (GPC). No alumina can be used if TBBP-A is to be analysed, lipid removal is by GPC.

2. Principles of the Method

Samples are extracted by pressurised liquid extraction (PLE) using a Dionex Accelerated Solvent Extractor (ASE 300) under temperatures and pressure. Solvent is added to an extraction cell and heated before being flushed into a collection bottle. 5% Deactivated alumina can be added to the extraction cell to remove lipid from biota samples (HBCD analysis only).

3. Reference Materials

In each batch of samples (maximum 12) a spiked sample (Cod liver Oil) and a procedural blank must be analysed.

4. Reagents

Anhydrous Sodium Sulphate (Analar, granular).
iso-Hexane (HPLC grade with bakelite caps, Rathburn Chemicals).
Acetone (HPLC grade with bakelite caps, Rathburn Chemicals).
Alumina (Aluminium oxide 9 standardised) – Merck 1.01097.1000 .
Distilled water – ultra pure.

If an alternative supplier is used, an equivalent grade of reagent is used.

5. Equipment

Dionex Sample Cells (various sizes)
Dionex glass fibre filters
Dionex cell filter insertion tool
Sample collection bottles
Balance (set to read to 3 decimal places)
Glass Beakers (various sizes)
Glass jars and lids (250 ml)
Spatulas
Forceps
Aluminium foil
Solvent reservoir bottle
Dionex ASE 300 (EN 1241)
Calibrated syringe
Ultra sonic bath
Drying oven (EN 547)
Measuring cylinders (various sizes)
Glass stirring rod
Test tubes
6. **Environmental Control**

The extraction process is carried out on the bench in the spark proof room (Room 505) with the fume cupboards in operation. Measuring and transfer of solvent is undertaken in the fume cupboard. Lab coats must be worn at all times in labs 505 and 506. The Dionex ASE is cleaned daily by both Acetone and iso-hexane before use as per SOP 0371. Ensure the ioniser is switched on.

7. **Interferences**

HBCD and TBBP-A analysis requires a stable environment, avoiding contamination of samples and reagents e.g. contact with fingers, dirty equipment. Glass fibre filters are only handled with forceps. All glassware/extraction cells are solvent washed with either acetone or iso-hexane prior to use. Nitrile gloves are to be worn while working in the laboratory. The addition and transfer of extracts and standards is by means of either glass pipette tips or calibrated glass syringe to prevent contamination.

Ensure the air purifier is switched on to reduce possible contamination from particulates in the air.

8. **Sampling and Sample Preparation**

All biota samples should be defrosted at room temperature or in the fridge overnight and exposure to direct sunlight or heat should be avoided.

9. **Analytical Procedure**

9.1 **Cleaning of equipment/materials**

9.1.1 **Glass fibre filters** - Glass fibre filters are wrapped in aluminium foil and placed in a muffle furnace, set to 200°C. The filters are muffled for at least 12 hours. Date, time in/out of furnace, temperature and operator are recorded at the back of the Dionex ASE logbook, which is kept next to the instrument.

9.1.2 All extraction cells, caps and collection bottles are solvent washed with acetone or iso-hexane followed by iso-hexane, with the latter being allowed to evaporate. The collection bottles lids are fitted with ultra clean low bleed septa. Cells and caps are numbered 1 – 12 (s, small, m, medium and L, large). Ensure each cell is fitted with the appropriately numbered lid.

9.2 **Sample preparation**
9.2.1 Biota sample preparation.

9.2.1.1 Ideally the % lipid of biota samples should be determined first using the Smedes method (M 0890). If not then use the indicative values in the Table 2, determined on various biota types. For HBCD/TBBP-A analysis an appropriate amount of sample equivalent to 300 mg of lipid is used in the extraction.

Approximate weights and indicative % lipid for various tissue samples:

<table>
<thead>
<tr>
<th>Biota type</th>
<th>weight</th>
<th>Indicative % lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish muscle, lean</td>
<td>10-15 g</td>
<td>3%</td>
</tr>
<tr>
<td>Fish muscle, fatty</td>
<td>0.5 - 2 g</td>
<td>15%</td>
</tr>
<tr>
<td>Fish liver</td>
<td>0.5-3 g</td>
<td>40%</td>
</tr>
<tr>
<td>Mussel</td>
<td>8-12 g</td>
<td>2.5%</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>0.1-0.3 g</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 1 – Indicative values to be used if % lipid has not first been determined.

Label both the side and lid of a glass jar with the UKAS ID. The sample is weighed in a glass jar and the weight recorded in the organic Worksheet B 638. Individual samples are cut into small pieces using solvent washed, forceps, scalpel or scissors. Pooled samples may have been previously homogenised (SOP 130). Add 200 ul by means of calibrated syringe of the internal standard prepared as per SOP 0600.

The remainder of the sample should be returned to the deep freeze as soon as weighing of all samples in the batch is completed.

9.2.1.2 Solvent washed sodium sulphate (SOP 1643) is added to the sample to allow drying. Use between 20 and 40 g of sodium sulphate. Mix the sample and sodium sulphate using a solvent washed spatula.

9.2.1.3 The spatula used to mix the sample with sodium sulphate should be rinsed down into the jar with iso-hexane (2 x 1ml).

9.2.1.4 Each batch consists of a procedural blank, spiked sample (Cod Liver oil) and up to 10 samples.

Repeat steps 9.2.1.1-9.2.1.3 for the remaining samples, blank and spiked sample.

Spiked sample
Cod liver oil (≤ 300 mg) is spiked with calibration solution F (nominal 0.4 μg ml⁻¹, 500 μl). The spiked cod liver oil is treated as a sample.

Procedural blank
The procedural blank consists of only 30 ± 5 g sodium sulphate.
The forceps, scalpel and scissors are rinsed with acetone and dried with blue roll between samples. The waste acetone is emptied into a non-chlorinated solvent waste bottle.

**9.2.1.5** Place lid on glass jar and store overnight in a refrigerator.

**9.2.1.6** Remove from the fridge and grind with a pestle wrapped in aluminium foil for at least 2 minutes.

### 9.3 Filling of extraction cells

**9.3.1** Samples are typically extracted using the 100 ml extraction cells. 
**Note** It is permissible to use an alternative size of cell if necessary as long as the spiked sample and procedural blank are extracted in the same size cells.

**9.3.2** Cells and caps are numbered, ensure cap and cell body numbers correspond. Cells are filled as per schematic Figure 1.

**Figure 1** Schematic of PLE cell for the extraction of HBCD and TBBP-A from biota.

**9.3.3** Unscrew the top cap from the cell body. Place 2 filters in the cell at a slight angle (Dionex ASE 300 manual 3-6). Place the insertion tool over the filters and slowly push the insertion tool into the cell. Ensure the filter is in full contact with the cell.

**Note:** Do not place the filter in the bottom cap before installing the cap, this creates an improper seal and allows leaks.
9.3.4 To the cells add solvent washed sodium sulphate (SOP 1643), 10 ± 1g via a funnel.

- HBCDs extraction alone from biota only - Add 5 ± 0.1g, via a funnel, of 5% deactivated alumina (SOP 0430)

- TBBP- A extraction **DO NOT ADD ALUMINA**.

Place another filter in the cell and push down on to the alumina, using the insertion tool (HBCD only). No filter paper is added if TBBP A is to be analysed.

Add the sample/ sodium sulphate to the cell (see section 9.2.1) via a funnel.

Add a small amount of sodium sulphate to the jar and stir around before adding this to the cell via the funnel.

Rinse sample jar with 5 ml of iso-hexane then add this carefully to top of the sodium sulphate.

Add more sodium sulphate to fill any void volume remaining.

The whole cell is tapped down using the insertion tool – top up with sodium sulphate if necessary before adding a further filter on top before hand tighten the top and bottom lids of the cell.

**DO NOT USE A WRENCH OR OTHER TOOL TO TIGHTEN THE CAP.**

9.4 Proceed to extraction by PLE SOP 0371.

10. **Calculation of Results**

   Not relevant

11. **Precision, Accuracy and Practical Detection Limits**

   Not relevant.

12. **Results**

   Not relevant

13. **Safety**

   AI 157
1. **Introduction and Scope**

   This SOP describes the clean-up, preparation and liquid chromatography-mass spectroscopy (LC-MS) procedures required for the determination of hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBP-A) in biota.

2. **Principle of the method**

   The lipid material is separated from HBCD/TBBP-A using gel permeation chromatography (GPC). The solvent is removed and the residue reconstituted in methanol prior to analysis by LC-MS.

3. **Reference materials**

   Not Relevant

4. **Reagents**

   Dichloromethane (DCM), water, acetonitrile, methanol, acetone and iso-hexane will be HPLC grade, Rathburn Chemicals Ltd.

   Ammonium acetate puriss for MS > 99% Sigma-Aldrich

5. **Equipment**

   Phenomenex phenogel column (300 x 21.2 mm, 50Å)
   Syncore Analyst evaporator
   Rotary Evaporator
   Spectra Physics HPLC pump and Rheodyne injector
   ½ dram vial (1.75 ml)
   Calibrated timer
   2.5 ml syringe (non-calibrated)
   100 ml measuring cylinders
   100 ml pear shaped flasks
   Pasture Pipette
   Pipettor (not calibrated)
   Beakers
   Volumetric flask (100 and 1000 ml)
   Filter funnel
   HPLC column – Luna 5μ C18 100 Å 150 x 2.00 mm (Phenomenex UK)

6. **Environmental control**

   See individual SOPs

7. **Interferences**

   Not Relevant
8. Sampling and sample preparation

Not Relevant

9. Analytical procedure

9.1 Preparation of sample for GPC

9.1.1 Samples are extracted using by PLE as per SOP 0605. The iso-hexane PLE extract is transferred to solvent washed Syncore tubes with iso-hexane washings (2 x 10 ± 2 ml). The solution is evaporated by Syncore evaporation (SOP 1640) to ~ 0.5 ml.

9.1.2 The solution is transferred with washings, by means of pasture pipette and pipettor, to a ½ dram vial. If the volume of the vial is exceeded, excess solvent can be removed under a stream of scrubbed nitrogen.

9.2 GPC clean-up

9.2.1 The DCM/iso-hexane mobile phase is prepared by measuring 500 ± 10 ml of iso-hexane using a measuring cylinder, followed by 500 ± 10 ml of DCM. Both solvents are transferred into a 1 L Duran bottle, shaken vigorously for at least 30 seconds and sonicated for 10 ± 2 minutes before use.

The flow rate of the HPLC pump is set at 2 ml min⁻¹. The flow is checked before use by measuring the volume eluted from the column in 10 minutes ± 10 secs following a 20 ± 5 minute settling period. The volume should be 20 ml ± 1 ml, if not the GPC column should be cleaned (9.3.1)

9.2.2 The iso-hexane entire extract (~1.5 ml) is injected onto the column using a 2.5 ml syringe via a rheodyne valve and a 2 ml loop. The first fraction collected (~150 ml, volume determined in 9.3) is discarded. The fraction containing HBCD/TBBP A (~160 - 250 ml, exact elution volume determined in 9.3) is collected in a measuring cylinder.

9.2.2 Approximately 50 ml of the GPC eluate is poured into a 100 ml pear shaped flask and concentrated to a small volume (~10 ml, note this volume does not have to be exact and is not critical to the procedure) by rotary evaporation. The remaining ~ 40 ml of eluate is added and the solution concentrated to dryness.

Note: The temperature of the water in the water bath should be ≤ 35 °C.

9.2.3 Methanol (0.5 ml) is added to the residue using a 1 ml calibrated syringe. The solution is mixed using a whirli-mixer for at least 30 seconds. The solution is transferred using the syringe to an HPLC vial. To the pear shaped flask is added a further 0.1 ml of methanol, which is swirled gently by hand before being combined with the 0.5 ml in the HPLC vial. The sample is analysed by electrospray LC-MS as per 9.4.

9.3 Determination of HBCD/TBBP-A GPC fraction
9.3.1 Every 12 samples the GPC column is cleaned and the HBCD/TBBP A fraction is checked. The samples run and split check volumes are recorded on record sheet B77. To clean the column, the mobile phase is first changed to acetone and run for 30 ± 5 minutes at 2 ml min⁻¹. Next the mobile phase is changed to methanol and run for 30 ± 5 minutes, followed by acetone for 30 ± 5 minutes. The mobile phase is finally changed back to DCM/iso-hexane (1:1, v/v) and run for at least 2 hours before injecting a sample.

9.3.2 The HBCD/TBBP-A fractionation is checked every 6th column clean. To check the HBCD/TBBP-A fractionation, 1000 µl of the stock solution (in methanol) is injected into the GPC column using a 1 ml calibrated syringe. The flow rate is set at 2 ml min⁻¹. The following fractions are collected in 100, 10 and 5 ml measuring cylinders: 0 - 150 ± 0.5 ml, 150 - 160 ± 0.5 ml, 160 - 170 ± 0.5 ml, 170 - 240 ± 1.0, 240 - 250 ± 0.5 ml, 250 - 260 ± 1 ml, 260 - 270 ± 1 ml, 270 - 350 ± 1 ml.

9.3.3 The fractions are transferred with washings to pear shaped flasks and concentrated to a small volume by rotary evaporation. iso-Hexane (10 ± 1 ml) is added by means of a measuring cylinder and the extract concentrated to ~0.5 ml by rotary evaporation. The extract is transferred to a GC vial and analysed by GC-ECD.

9.3.4 GC-ECD; The fractions are analysed on the PE Clarus using GC method CLBFRtest;

Carrier (Hydrogen): split flow 10.0 ml min⁻¹
Detector B gas flow: 17 ml min⁻¹

Column: HP 5 or equivalent
60 m
Autosampler settings:

Injection volume: 0.5 µl
Sample washes: 0
Sample pumps: 6
Solvent B washes: 6
Viscosity delay: 0
Injection speed: normal

Temperature program:

Initial temp: 80°C
Initial hold: 1 min
Ramp: 15°C min⁻¹ to 150°C, 10°C min⁻¹ to 250°C, 5°C min⁻¹ to 280°C
Hold: 20 min
Total run time: 42 min
Equilibration: 2 min
Detector 320°C
Injector 120°C

The sequence is setup as per SOP 1241
9.4 Determination of HBCD/TBBP A by LC-MS

9.4.1 Preparation of mobile phase

9.4.1.1 Mobile Phase A - Ammonium acetate (0.771 ± 0.001g) is accurately weighed into a glass beaker. To this is added water (20 ml ± 1 ml) and the solution gently swirled before decanting via a filter funnel to a volumetric flask (1000 ml). Aliquots of water (20 ± 1 ml) are added to the beaker, with swirling, and decanted into the volumetric flask a further 3 times and made up to volume with water. The solution is shaken before transferring to a labelled Duran bottle and marked with an expiry date of week from the date of preparation.

9.4.1.2 Mobile Phase B - Ammonium acetate (0.771 ± 0.001g) is accurately weighed into a glass beaker. To this is added water (20 ml ± 1 ml) and the solution gently swirled before decanting, via a filter funnel, to a volumetric flask (100 ml). Aliquots of water (20 ± 1 ml) are added to the beaker, with swirling, and decanted into the volumetric flask a further 3 times and made up to volume with water. The solution is shaken before transferring via a filter funnel to another volumetric flask (1000 ml) and made up to volume with acetonitrile. The solution is shaken before transferring to a labelled Duran bottle and marked with an expiry date of week from the date of preparation.

Note; Record the ammonium acetate weight, lot number and balance used in lab book.

9.4.2 LC-MS Setup

9.4.2.1 The electrospray ion source is used for the analysis. Carry out start-up procedure (SOP 1715).

A Duran bottle with methanol is placed in the solvent reservoir tray. To flush the lines out, the solvent lines (A & B) are placed in the methanol, and the HPLC set to 50% and 50% at 0.250 ml min⁻¹ for both A and B mobile phases for at least ½ hour. After half an hour, the methanol may be removed and both A and B mobile phases placed in the solvent reservoir tray. Aqueous phase (A) is placed on line A and solvent based phase (B) is placed on line B. The purge valve is opened and the two lines are set to 50% and 50% at 5 ml min⁻¹. The pump is allowed to purge for at least ten minutes. The purge valve is closed and the system is flushed with A (60%) and B (40%) at 0.250 ml min⁻¹ for at least five minutes with no column attached. The column is attached and mobile phase is allowed to pump through the column for at least twenty minutes.

Note: The eluants should be collected in a beaker and disposed of to non-chlorinated waste.

9.4.2.2 Ensure LC method is set up in the AQUIRE MODE for HBCD/TBBP A analysis (AI 2006, BFR_opt_sim_160707.dam) as follows;

- **Duration:** 45 mins
- **Scan Type:** Negative Q1 Multiple Ions
- **Resolution Q1:** Unit
- **MR Pause:** 5.00 ms
- **MCA:** No
- **NEB:** 12.00
Cur: 10.00
TEM: 350
IS: -2000
Step size: 0.00 amu

Ions: 543.1, 540.9, 641.0, 639.1, 553.2, 554.8, 658.2

Dwell time: 250 msec

Gradient:

<table>
<thead>
<tr>
<th>Total time (min)</th>
<th>Flow rate (μl/min)</th>
<th>A %</th>
<th>B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>10.0</td>
<td>250</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>32.0</td>
<td>250</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>40.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>45.0</td>
<td>250</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Maximum Pressure: 290 psi
Compressibility: 75
Dead volume (μl): 40
Maximum Flow Ramp (ml/min²): 100
Injection volume (μl): 25.00
Sample Speed (μl/min): 200

9.4.2.3 The sequence is created as per **SOP 1720**. A set of calibration standards is analysed with each batch of samples. The calibration standard is prepared as per **SOP 600**.

10. **Calculation of results**

See **SOP 1710**

11. **Precision, accuracy and practical detection limits** -

Recoveries- See Method **M660**

12. **Reports** -

Not relevant

14. **Safety** - Refer to assessment number **AI157**

15. **Literature references**

not relevant