Analysis of Hexabromocyclododecane (HBCDD) in Environmental Samples by Liquid Chromatography Mass Spectrometry

MRes Applied Analytical Science (LCMS)

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Abstract

Hexabromocyclododecane (HBCDD) is a brominated flame retardant that has been identified as a priority substance within the European Water Framework Directive and must be monitored in the environment by regulatory bodies such as Natural Resources Wales. This project investigated the extraction and analysis of the three main isomers of HBCDD in different matrices including freshwater, saline and biota, exploring different compound extraction techniques to find the optimal procedure for each matrix investigated and finding the optimal parameters for analysis.

A method for analysis of aqueous saline and freshwater matrices was developed and validated involving an extraction procedure using hydrophilic-lipophilic balance (HLB) solid phase extraction (SPE) disks, elution into methanol and a x1000 concentration of the sample extract. Analysis was conducted via liquid chromatography tandem mass spectrometry with a quadrupole mass spectrometer operating in negative electrospray ionisation mode. The target limit of quantitation values for each isomer of 75 pg/L and 150 pg/L for saline and freshwater matrices respectively was achieved in both matrices, with % accuracies for both matrices ranging from 97.3 – 102.7% and every isomer in both saline and freshwater fell below the precision target of <15% RSD. Initial method development on biota matrix involved research of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) kits with alternative dispersive SPE kits tested, and investigation into homogenisation techniques of the biota matrix.

The result of this project was implementation of a fully validated method for extraction and analysis of HBCDD in saline and freshwater matrices as a routine monitoring test of HBCDD abundance in the environment. Further work will need to be conducted on the analysis of HBCDD in biota matrix.

Contents

1. INTRODUCTION

1.1 Environmental pollution

Environmental pollution is the contamination of air, land or water by a substance that is either inherently toxic in nature or is persistent, in that it does not easily break down and so can accumulate to concentrations that present a hazard to the environment. The consequence of this pollution can be to damage or destroy the environment in which it occurs, or to hinder a beneficial effect that the environment provides. Water pollution can be categorised depending on the type of water source. Surface waters comprise of oceans, rivers, and lakes. Ground waters are found in underground rock structures such as aquifers. Some of the most fundamental uses of groundwater is to provide us with a supply of drinking water and to also feed our irrigation systems. The contamination of these water sources can result in a biomagnification of harmful substances via the food chain as well as direct contact with contaminated drinking water sources. In 2000, European Parliament created a strategy called the Water Framework Directive (WFD) to combat the pollution of water, which involved identifying substances that 'pose a significant risk to, or via, the aquatic environment' and created the first list of what we know as 'priority substances' and originally consisted of 33 of these pollutants[1].

1.1.1 Current regulation

The Water Framework Directive (WFD) is legislation with the primary goal of remediating Europe's polluted waters and ensuring they maintain a good water quality standard. The maximum concentrations of the priority pollutants (substances) are outlined in amended legislation, forming the Environmental Quality Standards Directive (EQSD). Priority Hazardous Substances are categorised as being persistent, bioaccumulative and toxic and are identified using criteria outlined in the Technical Guidance Document for Risk Assessment in support of Commission Directive 93/67/EEC of 20 July 1993, which gives the principles for assessing risk both to people, and to the environment[2]. In 2013 the original directives were amended further to include 12 new chemicals that offer potential risk to water quality and have been added to the list of Priority Hazardous Substances, for monitoring and control in EU surface waters[3]. These have been assigned concentration limits that must be upheld for a body

of water to achieve 'good chemical status' and are outlined in EQSD. Two ways of measuring this concentration are as follows:

> "A threshold for the average concentration of the substance concerned calculated from measurements over a one-year period. The purpose of this standard is to ensure protection against long-term exposure to pollutants in the aquatic environment;
>
> A maximum allowable concentration of the substance concerned, i.e. the maximum for any single measurement. The purpose of this standard is to ensure protection against short-term exposure, i.e. pollution peaks."[4]

These criteria are monitored by the regulatory bodies of the member states, e.g., Natural Resources Wales Analytical Services (NRWAS), where samples are collected, prepared, analysed as routine monitoring programmes to protect environmental and public health.

1.1.2 Persistent organic pollutants

'Persistent organic pollutants' (POPs) is a term used to describe synthetic organic compounds in the environment that do not break down very easily or very quickly. Due to this resistance to break down, they have the potential to accumulate both by being continually introduced to the same source, or by entering the food chain and bioaccumulating with each trophic level. POPs started to be commonly used in the 1940's, when a post-war boom in industrial production produced a market for many of these chemicals that had a range of advantageous characteristics for crop production, pest control, and industry. There were also POPs that were inadvertently created during various industrial processes. Unfortunately, the impact these would have on the environment and on human health was not realised until years later.[5]

One prominent sub-group of POPs are brominated flame retardants, or BFRs. The mechanism for these compounds is to slow or completely stop the propagation of a fire by suppressing the chemical reactions taking place within the flame[6]. Halogenated flame retardants repress combustion mainly by a radical mechanism, disrupting the exothermic process. Bromine is the most efficient halogen for flame retardation as the liberation of hydrogen bromide (HBr) is believed to occur over a narrow temperature range and so would be available in higher concentrations than, for instance, chlorinated flame retardants, that will

form hydrogen chloride (HCl) radicals over a much broader range of temperatures, and so will be available at lower concentrations[7]. See Figure 1.0 below for the interaction mechanisms for this process.[8] Hexabromocyclododecane is a highly stable halogenated aliphatic cyclic hydrocarbon making it very persistent in the environment. It has a high bio-concentration factor with human half-life varying between 165 days for α-HBCD and an estimated 55 days for the β- and γ-isomers.[9] HBCDD is categorized as a persistent organic pollutant (POP) because of its persistence, toxicity, and ability to bioaccumulate.

$$HBr + OH\bullet \rightarrow H_2O + Br\bullet$$
$$HBr + \bullet O\bullet \rightarrow OH\bullet + Br\bullet$$
$$HBr + H\bullet \leftrightarrow H_2 + Br\bullet$$
$$HBr + RCH_2\bullet \leftrightarrow RCH_3 + Br\bullet$$
$$RBr \leftrightarrow R\bullet + Br\bullet$$

Figure 1.0: Interaction mechanism between bromine and the free radicals produced during a fire

Hexabromocyclododecane (HBCDD) is a group of polybrominated flame retardants (BFRs) used in building materials and electronics.[10] HBCDD is known as an additive BFR, meaning that the compound is blended in with the polymer being treated as opposed to it being chemically bonded, resulting in a far higher chance of it leaching out of the product[11].

This chemical has 16 possible isomeric structures, each with different reactivity, resulting in challenging analysis conditions with methods needing high levels of selectivity to distinguish these isomers. The primary compound, 1,2,5,6,9,10-HBCDD, in addition to its 3 most abundant diastereoisomers, α-HBCDD, β-HBCDD and γ-HBCDD (see Figure 1.1 below), have recently been included as a Priority Hazardous Substances following the association with several chronic adverse effects in humans after exposure through diet and inhalation of contaminated dust particles[3][12]. These compounds are highly stable in the environment with reasonable water solubility (48.8, 14.7 and 2.1 μg/l for α, β and γ–HBCDD, respectively); the estimated half-life of 51, 1440 and 5760 hours in air, water and sediment respectively, and hydrophobicity (log K_{ow} of 5.6)[13] facilitating its bioaccumulation and long-range atmospheric transport[14]. These characteristics can pose additional problems for the

environment with the latter believed to be the primary route for HBCDD introduction into marine waters[15]. For marine animals, a major increase in malformity and mortality have been observed; a study on the toxicity of the three main stereoisomers on zebrafish embryos showed that exposure to HBCDD caused significant delays in the embryo hatching, depressed heart rate in the larvae as well as growth inhibition[16].

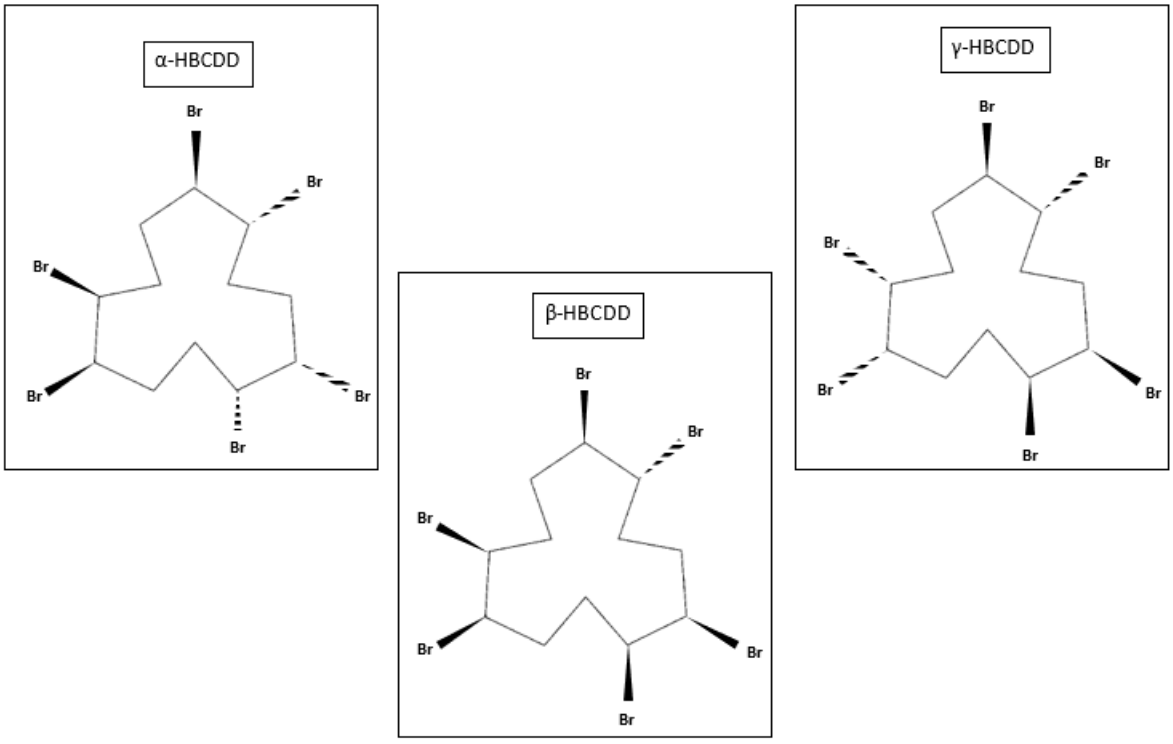


Figure 1.1: Chemical structures of α, β and γ – HBCDD, the most abundant stereoisomers[17]

1.2 Analytical methods for measuring environmental pollution

Many environmental pollutants are routinely monitored by laboratories such as NRWAS and the type of analysis is highly dependent on the nature of the analyte of interest. Due to this, the laboratory is split into two main areas of pollution analysis: organic and inorganic. The inorganic section analyses for pollutants such as phosphate and nitrate, whereas the organic section analysis includes groups of compounds including pesticides, herbicides and flame retardants. Inorganic pollutants are easier to determine and once a method has been developed to analyse these molecules, it is therefore unlikely to change, and the method

remains robust. Organic molecules however are more difficult to determine, and method development can involve a little more trial and error to discover what will and will not work with some of the more complicated molecules.

There are certain characteristics of the analyte that need to be considered when considering an appropriate analytical method. These can be whether the molecule is thermally labile and breaks down at high temperatures, if the molecule is polar or non-polar, if the molecule is pH sensitive, and the chemical structure of the compound.

1.2.1 Sample preparation

Aqueous matrix

There are many options to consider regarding the approach to sample preparation as techniques are continually being modified and improved where the primary goal is to increase the quality of the procedure and /or decrease the time it takes to complete that preparation protocol. A common simple extraction method is liquid-liquid extraction. This is very simple as it involves the mixing of two immiscible solvents and the partitioning of the analyte of interest. Other sample preparation techniques for aqueous samples include Soxhlet extraction, solid phase extraction and filtration.

Biota matrix

Sample preparation techniques for biota matrix have traditionally been long and arduous. The biota is often freeze dried before any further sample prep as this has a few advantages; the matrix is easier to homogenise after freeze drying, the sample can be stored for a far longer time after freeze drying , unlike fresh biota. This complete desiccation of the biota matrix may affect analyte recovery however and needs to be fully investigated before validation can take place.

1.2.1.1 QuEChERS – Quick, Easy, Cheap, Efficient, Rugged, Safe

QuEChERS is a novel analytical approach to solid phase extraction which has simplified the process of separating analytes of interest from the matrix. The name is an acronym of Quick, Easy, Cheap, Efficient, Rugged, and Safe. The original process, depicted in Figure 1.2 below, involves a simplified version of liquid-liquid extraction, where acetonitrile

and magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) are added to the sample in a centrifuge tube which is then shaken vigorously to encourage partition of the analyte to the solvent; the sample is then centrifuged to separate the salts from the acetonitrile. An aliquot of the solvent layer is removed from the rest of the sample and undergoes a clean-up stage by dispersive solid phase extraction (dSPE), which involves adding of a mix of the clean-up materials $MgSO_4$ and primary secondary amine (PSA) which remove many polar matrix components, then agitating the sample and centrifuging, giving a final extract for analysis[18]. The QuEChERS method will be explored for analysis of HBCDD in biota.

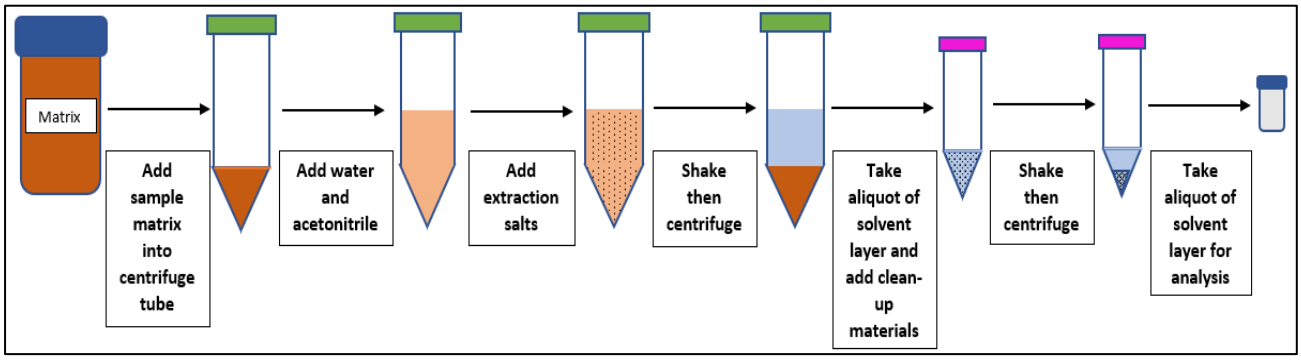


Figure 1.2: Illustrative representation of QuEChERS procedure[19]

1.2.1.2 Liquid Chromatography

Liquid Chromatography (LC) is the process of separating a solution out into its individual eluting peaks, which is then analysed via a detector. This is achieved by injecting an aliquot of the solution of interest into the LC system and using a liquid solvent, called the mobile phase, to push it through a column, where the components are separated out via adsorption mechanisms with the column stationary phase. Originally, the movement of the mobile phase through the system acted via gravity flow, though now it is more common to use High Performance Liquid Chromatography (HPLC), where the mobile phase carrying the solution of interest is pumped through at an increased pressure of anywhere typically between 50 and 350 bar, resulting in a faster process and allowing a column containing far smaller particle size packing material to be used, increasing the degree of separation of components in

the solution. A reduction of the particle size means a larger surface area available for the analyte to interact with, also a larger number of the particles will fit inside the column which increases the number of potential paths the analyte can take to move through the column as well as distance of the flow path, allowing for a greater separation of analytes. Smaller particle size also reduces the amount of time it takes for the analyte to move in and out of the particle, reducing peak diffusion and resulting in better resolution. The analyte of interest is pushed through the column at a constant rate, which keeps the retention time constant each time the method is run, and the analyte within the injected aliquot is separated out from other compounds present in the solution by interactions with both the stationary phase of the column and the mobile phase pushing the analyte through the system. The separated analyte then elutes from the column and is carried through to the detector. Liquid chromatography was selected over gas chromatography, as previous published methods found that although total-HBCDD can be detected using GC, the individual isomers are not able to be analysed as they begin to interconvert at temperatures over 160°C[20]. This interconversion involves a change in the positioning of the bromine atoms of each isomer resulting in α-HBCDD being the predominant resulting isomer[21].

1.2.1.3 Instrumentation

The LC system is composed of stackable modules for the sample injection and tray, the pump, and the column oven. The mobile phase set-up for this method will include a binary pump system with an aqueous mobile phase and an organic mobile phase. Mobile phase is pumped through a built-in degasser to remove any excess gas in the liquid before continuing through the rest of the LC system, as gas bubbles in the lines can cause instability in the chromatography. The sample injector stack holds the sample vial, and the injector takes a pre-programmed volume of the sample and injects it into the flow path. The sample tray chamber is thermostat controlled, allowing the samples to stay at a stable temperature while waiting to be analysed. For larger injection volumes this temperature might be kept at a similar temperature to the mobile phase so that the infusion of sample into the mobile phase causes as little disruption as possible. With large injection, any temperature discrepancy between the sample and the mobile phase may cause a shift in retention time. However, for some methods it may be beneficial to keep the samples chilled slightly to maintain the analyte stability in the sample.

1.2.1.4 Practical considerations for optimisation

There are many considerations to make when choosing the optimal parameters for analysis. The chemistry of the analyte of interest is key to making these decisions. HBCDD is a non-polar compound and as such it is suited to reversed phase chromatography. This is where the initial mobile phase is polar and the stationary phase of the column in non-polar. HBCDD will be carried through the system by the mobile phase under pressure to the column, and then due to the non-polar nature of HBCDD, it will adsorb to the non-polar stationary phase of the column. As the method will utilise a binary pump system, the polar aqueous mobile phase will be partnered with a polar organic mobile phase. The ratio between the two mobile phases can be altered via a time programme in the software of the instrument, so during analysis the aqueous mobile phase can move the analyte to the column, and then the percentage of organic mobile phase can be increased to elute the analyte off the column, as the percentage of non-polar organic phase will eventually reach a concentration where the affinity HBCDD has for the column stationary phase will be transferred to the mobile phase, thus essentially pulling the analyte from the column. The efficiency of this elution is greatly influenced by the gradient and flow rate of the aqueous: organic mobile phases, a steeper gradient and/ or faster flow rate will cause HBCDD to elute more quickly from the column which has the benefit of a sharper chromatographic peak, but if it is eluted too quickly, the detector may not have sufficient time to accurately monitor the analyte which could result in a badly shaped peak due to a lack of data points across the peak. Conversely if the gradient is too shallow / flow rate is too low, and HBCDD is eluted very slowly from the column, the resulting chromatography could show a broad, shorter peak, which could potentially be further affected by other matrix components eluting at the same retention time. This ties into the Van Deemter equation, which is an explanation of column efficiency and is characterised by the following equation (1):

$$HETP = A + \left(\frac{B}{u}\right) + C_u \qquad (1)$$

Where:
HETP = Height equivalent to a theoretical plate
A = Eddy diffusion

B = Longitudinal diffusion
C = Resistance to mass transfer
u = Average mobile phase velocity

1.2.1.5 Resolution, capacity factor and retention

Resolution is the ability of a system to separate two components. There are 3 parameters that govern resolution: selectivity, efficiency, and retention. There is more than one way to calculate resolution, one being the following general resolution equation (2):

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'_2}{1+k'_2}\right) \qquad (2)$$

Where:
N = Column Efficiency, or number of theoretical plates in the column
α = Selectivity
k'2 = Capacity Factor for the later eluting peak of the pair

Column Efficiency

Column efficiency is the theoretical number of plates in a column, the higher this value the more efficient the column in relation to column particle size and flow rates.

There is a simple formula to calculate column efficiency (3):

$$N = 5.54(t_R \,/\, w_{05})^2 \qquad (3)$$

Where:
t_R = retention time
$W_{0.5}$ = width of peak at half height

Capacity Factor

Capacity Factor (k') is the measure of how much interaction an analyte has with the stationary phase of a column compared to the interaction it has with the mobile phase. A simple way to calculate this value theoretically is to multiply column ID by column length; however, the accuracy of this result would be affected in this case as the column used has superficially porous particles, and so have less volume than the calculations assume. A more accurate way of calculating the capacity factor is to find the zero time (t_0), which is the time at which a completely unretained compound will elute off the column; this allows for the calculation of capacity factor to take into account the column interaction only and removes any other factors affecting retention time to be taken out of the equation.

The formula to calculate Capacity Factor is shown below (4):

$$k' = [t_R - t(0)] \,/\, t(0) \qquad (4)$$

Where:
t_R = retention time of the peak
t(0) = retention time of an unretained peak

Selectivity Factor

Selectivity Factor refers to the degree of separation between two eluting peaks. It can also be defined as the ration of the capacity factors of two peaks, or (5):

$$\alpha = \frac{k'2}{k'1} \qquad (5)$$

Where:
k′2 = capacity factor of peak 2
k′1 = capacity factor of peak 1

Simple resolution equation

A more direct way to calculate resolution between two peaks is shown with the following formula (6) and depicted as an illustration in Figure 1.3:

$$R_s = 2\left(t_{R_2} - t_{R_1}\right) / \left(wb_1 + wb_2\right) \qquad (6)$$

Where:
t_R = retention time
w_b = width at base of peak
1 = first eluting peak
2 = second eluting peak

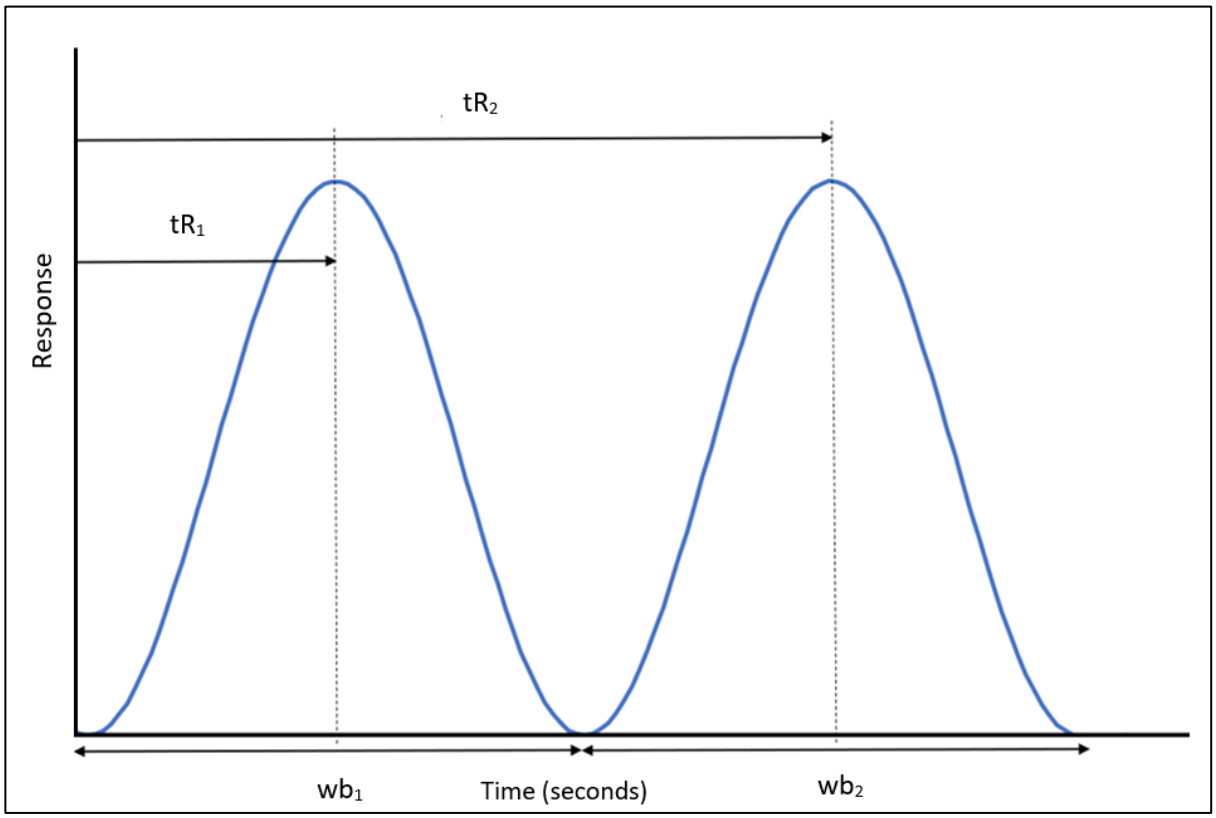


Figure 1.3: Visual representation of how to calculate resolution between two chromatographic peaks

1.2.2 Mass Spectrometry

Mass spectrometry is an analytical technique that identifies and quantifies the molecular mass of a compound by measuring the mass-to-charge (*m/z*) of the ionised species and produce

a mass spectrum that plots the abundance of each *m/z*. It is a common detector choice as the sensitivity and selectivity is good, it is capable of coupling with several different types of sample introduction systems, and the automation aspect of the technique allows for analysis on a large scale[22].

In the case of LC-MS, a sample is introduced to the instrument via a liquid chromatography system. Sample is injected into a liquid mobile phase, which is usually a mixture of organic and aqueous solvents that may have additional modifiers that influence the chemistry of the analyte of interest. The sample moves through an analytical column where chromatographic separation of analytes takes place; these are eluted off the column back into the mobile phase where they are transported and sprayed into the ionisation source of the mass spectrometer. The analyte is converted into ions in a gas phase. Ions are then directed into the mass analyser where they are sorted according to their *m/z* under a high vacuum. The ions then leave the mass analyser and enter the detector, where they are counted; some mass analysers will amplify each ion signal[23].

1.2.2.1 Instrumentation

There are a variety of options for the mass spectrometer system, with several ion sources available such as electrospray, atmospheric pressure chemical ionisation, electron ionisation and chemical ionisation. There are also many mass analysers now available, include the single quadrupole, triple quadrupole, time of flight and magnetic sector are a few of the more common ones available.

1.2.2.1.1 Ionisation sources

The ionisation source is the interface between the sample introduction system and the mass analyser. The analyte coming into the ionisation source must be desolvated as, in a gaseous state, it will be able to move freely through the mass analyser under vacuum. The analyte also needs to be ionised as the mass analyser will apply an opposing charge within the system to pull the analyte through. If the mass spectrometer is paired with an LC system, the liquid needs to be completely removed for the analyte

to travel through for analysis. There are different mechanisms for ionisation if an analyte, employed by different types of ion source. The two ion sources looked at for this project were the Electrospray Ionisation (ESI) source and the Atmospheric Pressure Chemical Ionisation (APCI) source. These are both examples of Atmospheric Pressure Ionisation sources, which are characterised by the ionisation of the analyte taking place under atmospheric pressure[24].

1.2.2.1.1.1 ESI

Electrospray transfers ions from solution to a gaseous state by the following mechanism: the sample solution passes through a stainless steel or quartz silica capillary that is maintained at a high voltage, usually somewhere between 2.5 – 6.0 kV, creating a potential difference which causes ionisation of sample droplets. The efficiency of this ionisation is further increased by application of a nebulising gas around the capillary, which aids the sample flow into the chamber. The build-up of charged droplets at the tip of the needle results in a potential gradient down towards the orifice of the analyser as the droplets are repelled from the concentration mass of like charge. The droplets are continuously reducing in size as this occurs, as the source temperature and application of a drying gas evaporates off the solvent. The continuous decrease within droplet surface area eventually reaches a critical stage where the surface area can no longer be maintained, and the analyte is released into a gaseous phase, whereby they are navigated into the analyser via a sampling skimmer cone[25]. This ionisation mechanism is more violent than that of APCI, and results in a higher degree of fragmentation of the analyte, providing a larger spectrum of *m/z* to use for analyte identification. See Figure 1.4 below for a visualisation of the mechanism of ESI.

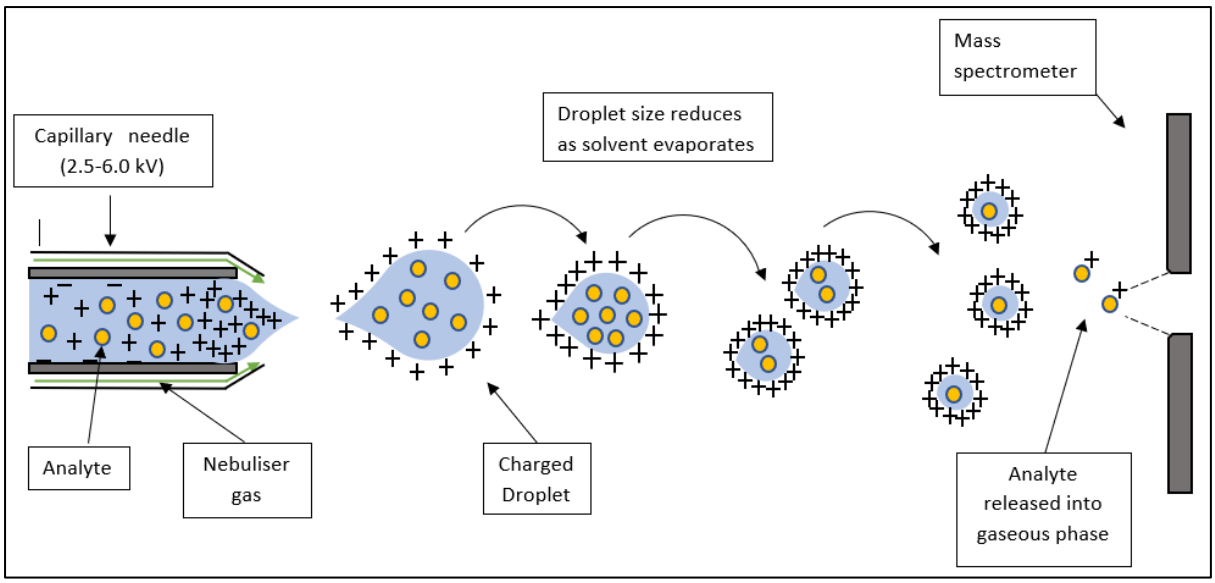


Figure 1.4: Schematic representation of the electrospray ionization process[26]

1.2.2.1.1.2 APCI

The mechanism of APCI ionisation differs to that of ESI, as the analyte is introduced into the ionisation source via a heated quartz tube and then pass by a high positive voltage corona discharge needle which produces N_2^+ and N_4^+. These ions collide with the passing solvent droplets, forming secondary gas ions such as H_3O^+, which then also collide with solvent ions, and eventually colliding with analyte ions creating a cascading effect of ionisation, with the increasing ion numbers increasing the ionisation efficiency. This is a very soft ionisation technique which results in very little fragmentation of the analyte ion, with the ion species analysed primarily being the molecular species with adduct ions[27]. See Figure 1.5 below for an illustration of the mechanism of APCI.

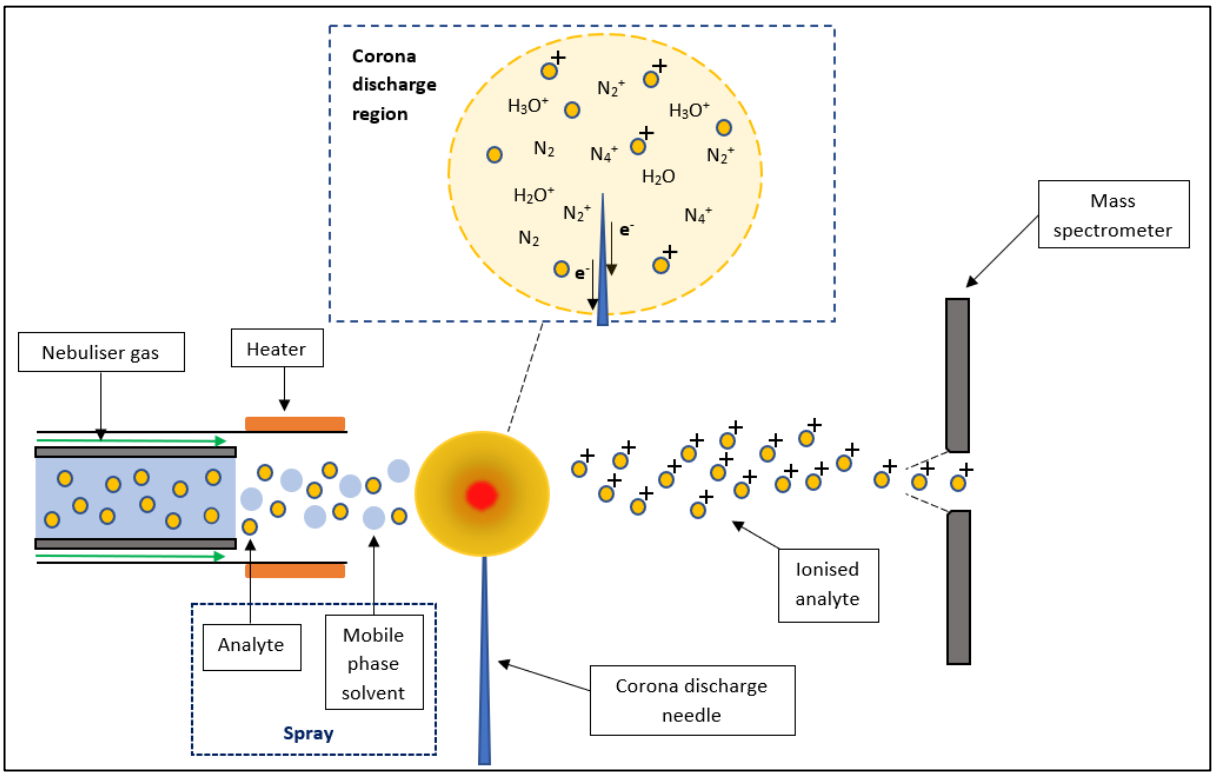


Figure 1.5: Mechanism of Atmospheric Pressure Chemical Ionization mass spectrometry[27]

1.2.2.1.2 Mass Analysers

For this project a triple quadrupole will be utilised alongside the electrospray source and the atmospheric pressure chemical ionisation source.

1.2.2.1.2.1 Triple quadrupole

The triple quadrupole mass spectrometer is named so because historically it contained three functioning quadrupoles. It is now more commonly known as a tandem mass spectrometer as the 'middle' quadrupole filter no longer functions a mass analyser and has since had the function of a collision cell. The additional quadrupole provides a number of new ways the analyser can be utilised, one useful feature being that the analyser now has the ability to detect the unique fragmentation pattern of both the precursor ion and the product ion that comes from it, thereby analysing for those two

m/z alone, significantly minimising background interferences and increasing sensitivity for the analyte[28].

1.2.2.2 Mode of operation for quantitation and data handling

This is performed via multiple reaction monitoring, or MRM, where the first quadrupole detects and delivers the *m/z* of interest to the second quadrupole, known as the collision cell, where it undergoes fragmentation. These fragments are then delivered to the third quadrupole, which will only be monitoring for the product ion *m/z* that was previously selected. The monitoring of both precursor and product ions provides an extra level of greater analytical specificity to the analysis and sensitivity, minimising the possibility of anything but the target analyte being identified as such. This method is far more robust than, for example, Selective Ion Monitoring (SIM) which looks for a selected product ion but does not include a precursor ion filter, which increases the probability of seeing a false positive result that can come from an interference that produces the same product ion fragment *m/z*[29]. Figure 1.6 below gives an illustration of the mechanisms for SIM and MRM.

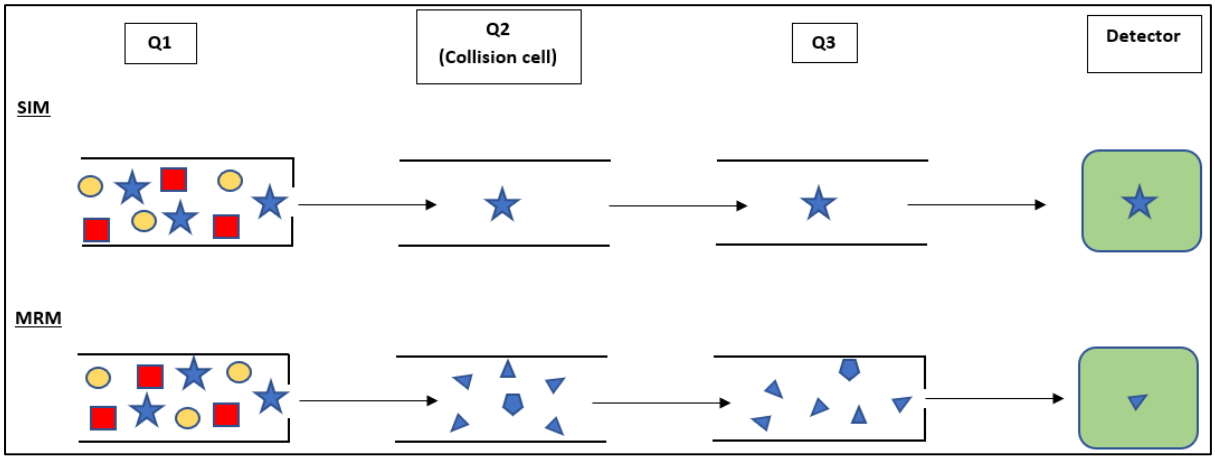


Figure 1.6: Illustration of the mechanism of SIM and MRM[30]

1.2.3 Method validation

Prior to analyte quantitation, the analytical method will first require method development, optimisation, and validation. The validation process involves testing the optimised protocol against benchmarks set out by professional standards and/or regulatory frameworks. The latter for environmental monitoring is audited by the UK Accreditation Service in accordance with ISO 17025[31]. This requires key parameters or figures of merit to be established, including: -

Precision and accuracy; precision being the closeness of results from repeated applications of the method to each other, regardless of how close they are to the target result. This should be performed as an inter-assay test on the same instrument in a short time interval and can also be tested with variables expected to occur during routine running of the method, such as running the method on different days, having the method performed by different operators and using different laboratory apparatus such as flasks, beakers and cylinders. Accuracy is the closeness of test results to the target result; this can be determined using a reference material, where a solution spiked with a known quantity of HBCDD undergoes the method in question and the result is analysed.

Limit of Quantification (LOQ) is the lowest concentration of the analyte of interest that is quantifiable (with those previously stated parameters being applied to an instrument); so, the chromatographic result of repeated samples is both precise and accurate, thereby giving confidence that the method is reliably consistent at this low level. There are parameters that can be applied to the method to allow for lower LOQ levels, one of these being the type of instrumental method used to acquire the data.

Linearity and Range – this is the capability of a method to show that it can relay an accurate concentration response over a representative range. It is represented as a calibration curve with standards at specific concentration intervals and is used to interpret the method validation sample results based on the surrogate standard recoveries of the samples in relation to the internal standard recoveries in the calibration curve standards. The samples for the calibration curve were not extracted via the same sample preparation techniques as the matrix samples; the internal standard used is a $^{13}C_{12}$ α-HBCDD, β-HBCDD, γ-HBCDD mix – so each isomer of interest has its own isotopically labelled standard assigned to it. Due to this, the calibration

curve used is not subjected to any issues that could arise with sample preparation procedures and can be viewed with confidence as a good example of a calibration curve.

Robustness

This element of method validation shows that the method can undergo minor, expected variations of parameters that are typical in real-life scenarios and still function sufficiently as a method. This can include parameters such as temperature, pump pressure and variations in mobile phase ratios. This level of testing gives confidence in the method reliability. Conversely, when testing parameters and finding that some variations do negatively affect the outcome of analysis, these can be included in the SOP as an indication of a fixed parameter that should not be changed. Instrumental parameters are routinely tested before analysis of each batch by way of a system suitability standard, which is a direct spike of each HBCDD isomer at the lowest calibration level plus the internal standard, and particular parameters are checked to ensure that the behaviour of the instrument has stayed within a particular specification – these parameters include the peak resolution between the two closest eluting peaks, the peak area, signal to noise, relative retention time, so the time between the elution of the internal standard compared to the native compound, and the full width of the peak at half the maximum height. These variations, having been investigated during the method validation stage, are able to be introduced as a permanent change in the method if needed, provided that the variation did not yield a negative outcome in the validation process. Any variations that were not part of the method validation process however cannot be introduced as a change to the method unless that change itself is validated on its own merit – if adjustments of any method parameters are found to improve the method, but these are discovered after method validation has been completed then another validation exercise to reflect the altered method must be performed before these changes can be incorporated into the method.

The robustness of the instrument method is the easiest to investigate as the parameters to test can be altered using software and so we can assume that the new parameters will be replicated accurately. The Shimadzu LC-8060 has a method optimisation software tool that will perform these variations after being given certain parameters such as a minimum and maximum value, and the interval values needed.

1.2.4 Previous methods of Analysis of HBCDD by LC-MS

Many current methods for analysis of HBCDD use complex sample preparation; one example involves analysing the presence of HBCDD in atmospheric particle samples via a 24-hour Soxhlet extraction, multiple evaporation steps, elution of separate sample fractions and solvent exchange [32] taking several days to complete. With many LC-MS techniques, particularly those using electrospray ionisation (ESI), ion suppression or enhancement can occur without appropriate sample preparation. Ion suppression occurs when molecules other than the analyte of interest but having a similar retention are present in large concentrations and elutes within the same time window. One of the mechanisms thought to be involved in ion suppression is the competition between the molecules for ionisation in the ion source, which then reduces ionisation efficiency[33]. However, sample preparation can be costly and time consuming, potentially resulting in sample contamination or analyte loss with each additional step. Other analytical methods such as gas chromatography mass spectrometry (GC-MS) are unsuitable for isomeric HBCDD monitoring due to poor compound stability at typical GC operating temperatures (>240˚C) and complex chromatography due to isomeric interconversion at temperatures >160˚C [20]. Therefore, despite challenges with ion suppression or enhancement, current analytical methods employ LC-MS using much softer, lower temperature conditions (e.g., ESI) offering enhanced sensitivity for HBCDD. A recently developed approach for pesticides for foodstuffs, known as the 'quick, easy, cheap, effective, rugged and safe' (QuEChERS) protocol, enables rapid sample preparation for solid and liquid matrices with minimal matrix effects at relatively low cost[34]. Recent work of the Godfrey research group (unpublished) has investigated this technique for a range of environmental matrices (including biota and wastewater) and successfully extracted persistent organic pollutants of interest to WFD with minimal matrix interference. However, to the best of our knowledge (and as part of the literature review for this thesis) this technique has yet to be tested for HBCDD and could provide an alternative method capable of extracting these matrices in less time and cost.

1.2.5 Research need

This project will underpin a validated method for the sampling, extraction and analysis of HBCDD isomers in the environmental matrices of interest to WFD for routine monitoring. To our knowledge, this project could lead to the first use of QuEChERS and passive sampling for HBCDD monitoring to inform future environmental and public health policy regarding the levels of HBCDD pollution. Based on the success of QuEChERS and passive sampling for other pollutants and matrices included in WFD, we anticipate this approach will provide a more rapid analysis and comprehensive data set of the levels of HBCDD at a location of interest.

1.2.6 Aim and Objectives

This research will investigate α-HBCDD, β-HBCDD and γ-HBCDD given their status as priority hazardous substances in different matrices (which could include fresh water, saline and biota) sampled from approved locations within Wales accessible by Natural Resources Wales (NRW). These will be prepared for analysis by liquid chromatography-mass spectrometry (LC-MS) and will require:

1. The development of an LC-MS protocol for quantitative analysis of the HBCDD isomers at a pg/L level and relevant sample preparation for each matrix investigated for analysis of the compounds.

2. A quantitative investigation of selected matrices for the chosen HBCDD isomers to understand the scale of the pollution.

2. MATERIALS AND METHODS

2.1 Chemicals, solvents, and apparatus

Tables 2.0 and 2.1 below show the necessary items needed for HBCDD method development to commence.

Table 2.0 : Chemicals required for HBCDD method development

Chemical	Manufacturer	Chemical formula	CAS number	Molecular weight
α-HBCDD 100 mg/L in acetonitrile	QMX	$C_{12}H_{18}Br_6$	134237-50-6	641.70
β-HBCDD 100 mg/L in acetonitrile	QMX	$C_{12}H_{18}Br_6$	134237-51-7	641.70
γ-HBCDD 100 mg/L in acetonitrile	QMX	$C_{12}H_{18}Br_6$	134237-52-8	641.70
Mixture of $^{13}C_{12}$-labelled α, β and γ-HBCDD 10 mg/L in toluene	Greyhound Chromatography	$^{13}C_{12}$ $H_{18}Br_6$	$^{13}C_{12}$ α: 870247-98-6 $^{13}C_{12}$ β: 870248-00-3 $^{13}C_{12}$ γ: 676552-82-2	653.61
Methanol	Romil	CH_3OH	67-56-1	X
Acetone	Honeywell	$(CH_3)_2$ CO	67-64-1	X
Isopropanol	Honeywell	C_3H_8O	67-63-0	X
Acetonitrile	Honeywell	C_2H_3N	75-05-8	X
Ultra High purity (UHP) water	In-house	H_2O	X	X
QuEChERS extraction kit (Original Method): 4 g $MgSO_4$, 1 g NaCl	Agilent	X	X	X
QuEChERS dispersive kit for general fruits and vegetables: 400 mg PSA, 1200 mg $MgSO_4$	Agilent	X	X	X
QuEChERS dispersive kit for fruits and vegetables with fats and waxes: 400 mg PSA, 1200 mg $MgSO_4$	Agilent	X	X	X
QuEChERS dispersive kit for drug residues in meat: 150 mg C18, 900 mg $MgSO_4$	Agilent	X	X	X

Table 2.1: Consumables and equipment required for HBCDD method development

Consumables and Equipment	Manufacturer
500 mL borosilicate measuring cylinder, grade A	VWR
500 mL borosilicate beakers	VWR
Hydrophilic Lipophilic Balance (HLB) Solid Phase Extraction (SPE) disks	Affinisep
Silanized 2 mL clear vials	Agilent
GF/C glass microfilters	Whatman
Positive displacement pipettors, 100 µL & 1000 µL	Rainin
Flip-flop tube assembly	Genevac
Diskcover-47 Disk holders	Restek
Glass block vacuum manifold	Thermo Scientific
Vacuum pump	Thermo Scientific
Rocket Synergy 2 centrifugal evaporator	Genevac
Reacti-vap nitrogen gas evaporator	Thermo Scientific
1L borosilicate wide neck sampling bottles	VWR
Agilent Poroshell 120 EC-C18 3.0 x 50 mm 2.7 µm column	Agilent
Agilent EC-C183.0 x 5 mm 2.7 µm column guard	Agilent

2.2 Instrumentation

Initial method optimisation for analysis of HBCDD in aqueous matrices was conducted using a Shimadzu Nexera X2 liquid chromatography (LC) system with a binary pump coupled to a Shimadzu 8060 triple quadrupole (QQQ) mass spectrometer. However, reproducibility tests conducted during the initial method development were not successful, with large responses occurring intermittently throughout the run. Due to irreproducibility of operation the optimisation of HBCDD measurement in aqueous and biota samples followed by the validation of the aqueous methods was conducted on an Agilent 1260 Infinity II LC system with a binary pump coupled to an Agilent 6495C mass spectrometer. The parameters chosen during optimisation of the HBCDD method on the Shimadzu instrument were fully transferable to the Agilent system, as there were differences in design of the systems, in particular the mass spectrometer – for example, the Agilent system uses nitrogen gas as the collision gas whereas the Shimadzu system uses Argon. However, the Agilent system was optimised for HBCDD analysis by the instrument manufacturer.

2.3 Aqueous sample preparation

The low concentration of HBCDD required for environmental monitoring would be a significant challenge for detection by LC-QQQ mass spectrometry. To mitigate this issue the sample preparation involved the pre-concentration of the analyte, raising the concentration to a level that can be confidently quantifiable. The analyte was collected from 500 mL of matrix water onto a solid phase extraction disk, eluted off the disk with methanol which was then evaporated down to a final volume of 0.5 mL, giving an overall concentration factor of x1000. The following steps give the finalised procedure for the extraction and analysis of HBCDD from freshwater and saline matrix.

Saline water matrix was collected from a saline collection point at Swansea University that is directly piped in from Swansea Bay and freshwater matrix was collected from a routine sampling point by a member of the NRW sampling team. Saline matrix was collected from Swansea Bay due to no routine sampling of saline samples being conducted when the matrix was required and was collected via an external tap before the matrix underwent any filtration processes within the University. Both matrices were collected in multiple 5 L sterile high-density polyethylene jerrycans, and approximately 50 L of each matrix was collected to ensure the same batch of matrix could be used through the validation in addition to the initial method development and a potential requirement to prepare extra batches. As part of the validation, these matrix waters were run as 'blank' tests, to observe the expected background for each. The matrix waters were stored in a routinely monitored cold room set at a temperature of 5±3°C.

2.3.1 Sample loading onto the HLB disk

On the day of analysis, the bottles were removed from the cold room and allowed to come to room temperature. The surrogate standard was also removed from the fridge and allowed to come to room temperature. A class A borosilicate measuring cylinder was used to measure out 500 mL of matrix water which was decanted into 500 mL borosilicate beakers. The surrogate standard was spiked into the sample bottles and a glass rod used to stir the spiked matrix.

HBCDD was extracted from the matrix using a solid phase extraction (SPE) method, where each sample matrix was passed through an Affinisep AttractSPE®Disk with a Hydrophilic Lipophilic Balance (HLB) sorbent. To hold the disk in place and allow for liquids to be passed through, Restek™ Diskcover-47 Extraction Disk Holders were used with the optional Diskcover-47 reservoir – Figure 2.0 below illustrates each component of the Discover-47 kit.

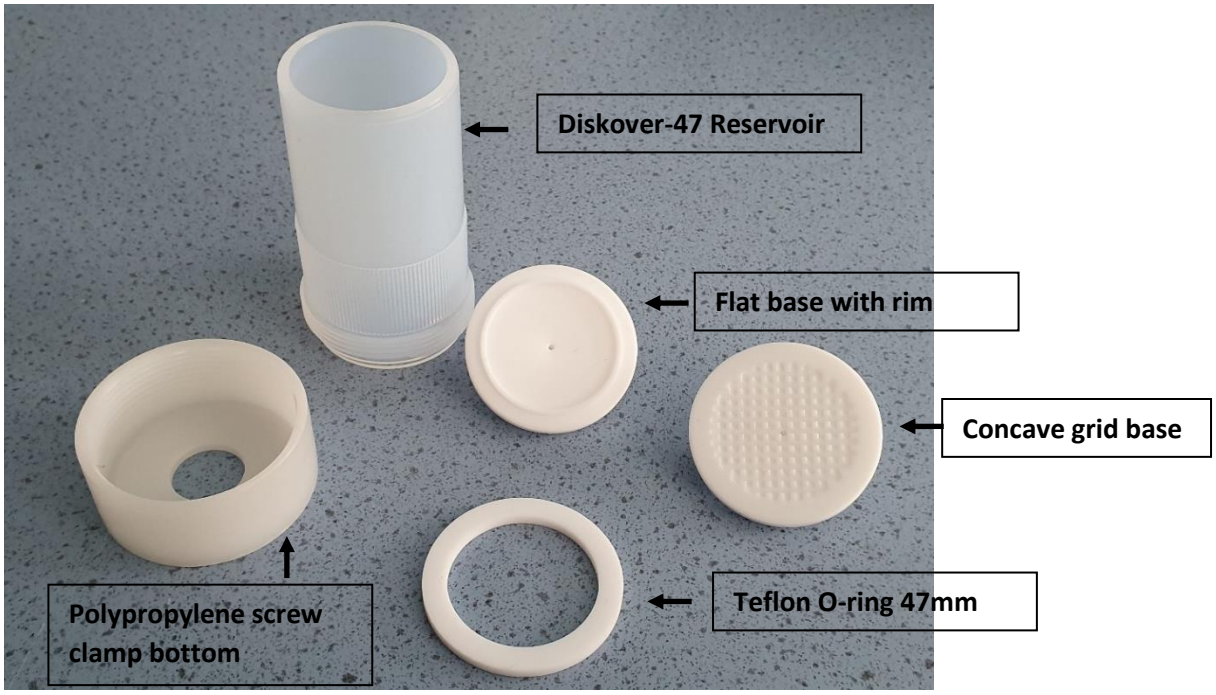


Figure 2.0: Restek™ Diskcover-47 disk extraction apparatus

For the first step in the sample preparation procedure the Extraction Disk Holders were assembled by placing the concave grid base into the polypropylene screw clamp bottom, placing a GF/C glass microfilter on the base first and then the Affinisep HLB disk on top. The concave grid base was necessary as the extra surface area was needed to help the 500 mL of sample move through the assembly at an acceptable rate, with the GF/C glass microfilter disk protecting the HLB disk from warping or tearing when a vacuum is being applied. A flat base was initially tested but it was found that sample moved through this at a very low rate compared to the grid base, as the grid provides space for the sample to 'pool' before moving through the centre nozzle. The Teflon O-ring was then placed on top of the disk, again to protect it from

tearing, and the reservoir was then screwed on. The Extraction Disk Holders were placed on an SPE vacuum manifold with adjustable taps to allow for control over rate the matrix moves through the disk.

Before use these disks were cleaned using the Affinisep method below to remove potential contaminants or interferences and conditioned to prime the stationary phase sorbent inside the disk. Step 4 was modified to allow for adjustment of the vacuum block tap to find an optimal flow rate.

1. Addition of 50 mL of acetone, left for 1 minutes and then vacuum applied to dry the disk.
2. Addition of 50 mL of isopropanol, left for 1 minutes and then vacuum applied to dry the disk.
3. Addition of 50 mL methanol, vacuum applied to draw approximately 1 mL through the disk, then close tap and allow disk to soak in methanol for 30 seconds. Draw the methanol through the disk under vacuum, but close tap while there is still a small amount of methanol on top of the disk.
4. Addition of 10 mL UHP water, draw water through and adjust tap to find a flow rate of approximately 1 drop/second. This rate should ensure that the sample does not pass through the disk too quickly, allowing all the analyte in the sample to interact with the disk. Ensure the disk does not go dry during this step, topping up with UHP water as needed.

Once this was completed the sample bottles were poured into the reservoirs, filling to approximately ¾ of the reservoir capacity and topping up as necessary until all the sample had passed through the HLB disk. The disks were washed by adding 50 mL of UHP water into each reservoir and applying a vacuum. The disks were then removed from the Extraction Disk Holder and the GF/C microfilter and placed onto a labelled tray lined with foil to completely dry overnight, as the presence of water in the final eluted extract was known to result in reduced sensitivity. The water may disrupt the quantitative elution of the analyte of interest off the trapping phase of the disk.

2.3.2 Elution and concentration of HBCDD

Cleaned and rinsed Extraction Disk Holders were used again in this step but only the flat bases were now used in the place of the grid bases as the GF/C microfilter was not used, to avoid the introduction of contamination when the elution solvent moved through the assembly. The dried disks were placed on the bases and the Extraction Disk Holders assembled as before and labelled with the appropriate sample name. The eluent was collected into double-ended ≈50 mL Flip-Flop tubes (see Appendix A) with a screwcap affixed to one end. These were rinsed with methanol, labelled, and placed into the vacuum box beneath the appropriate tap holding the corresponding Extraction Disk Holder. 30 mL of methanol elution solvent was added to each Extraction Disk Holder reservoir in three 10 mL increments, allowing each 10 mL to fully elute through the disk under gravity flow before applying the vacuum for 15 minutes after adding the final 10 ml to draw through any remaining solvent. The tubes were then removed from the vacuum box, the remaining parts of the Flip-Flop assembly were attached, and the tube was inverted to allow the silanised vial to be filled with the eluted solvent.

A Genevac Rocket Synergy 2 centrifugal evaporator was used as a time effective evaporative method as a vacuum is used to reduce the boiling point of the solvent and result in a faster evaporation time. The method used had the parameters shown in Table 2.2 below:

Table 2.2: Genevac Rocket Synergy 2 centrifugal evaporator parameters

Parameter	Stage 1	Stage 2
Pre-cool chamber	20°C	No
End stage On	Δt 30%	Time – 40 minutes
Drain at End Stage		00:06:00
Water control temperature	40°C	40°C
Inner control pressure	20 mbar	20 mbar
Stage time	02:30:00	00:20:00
Chiller set temperature	-10°C	

The eluent was evaporated down to approximately 0.5 mL and then adjusted to 0.5 mL by using a ‘check’ vial as a gauge which had 0.5 mL of methanol pipetting directly into it. The

'check' vial was the same vial type used to collect the final extract and so pipetting the same extraction solvent at the desired volume into an analogous vial was a sufficient way of ensuring accuracy of the final volume. If the solvent level was less than the required level then methanol was added to make the appropriate level, if more than the required level a Reactivap nitrogen gas evaporator was used to decrease the solvent level. The final extract was then ready for analysis.

2.4 Analytical method

The method for analysis of HBCDD was developed on an LC-QQQ system, as a multiple reaction monitoring (MRM) method, using the mass-to-charge (*m/z*) of the target precursor and selected product ions, to provide greater analytical specificity. The chromatographic separation involved an Agilent Poroshell 120 EC-C18 column with 3.0 x 50 mm, 2.7 Micron dimensions, using water and methanol mobile phases at a flow rate of 350 µL/min and a temperature of 50°C, with the gradient shown in Table 2.3 below:

Table 2.3: Gradient mobile phase conditions for LC separation where solvent A is UHP water and solvent B is methanol .

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	50.0	50.0	0.350	500.00
1.00	50.0	50.0	---	---
5.00	15.0	85.0	---	---
8.00	10.0	90.0	---	---
10.00	5.0	95.0	---	---
11.00	5.0	95.0	---	---
12.00	50.0	50.0	---	---

As HBCDD is non-polar, a starting gradient of 50:50 mobile phase was used as this composition would allow HBCDD to be sufficiently retained on the column. A later increase in organic mobile phase would then elute the analyte. Samples of 10 µL were injected on the column with a standard wash programme that flushed the injection port for 5 seconds. Once injected, the mass spectrometer acquired data in MRM mode using the conditions described in Table 2.4.

Table 2.4: Instrument parameters for HBCDD analysis method

MS Source Parameter	Value
Gas Temperature (°C)	160
Drying Gas Flow (L/min)	17
Sheath Gas Temperature (°C)	380
Sheath Gas Flow (L/min)	12
Capillary Voltage (V)	4500
Nebuliser (psi)	40
Nozzle (V)	800
iFunnel (Pressure RF)	H90 L100

Compound Name	Precursor Ion	MS1 Resolution	Product Ion	MS2 Resolution	Dwell (ms)	Collision Energy (V)	Cell Accelerator Voltage (V)
HBCDD	640.7	Unit	79.9	Widest	150	10	5
HBCDD	640.7	Unit	78.9	Unit	150	10	5

Two product ions were selected to have one act as a qualifier ion; as fragmentation of a precursor ion always produces the same fragmentation pattern, there should always be a specific ratio between the quantifier and qualifier ion. This gives extra confirmation that the product ion observed is from the analyte of interest.

The software used to control the instrument hardware parameters and collect the detection data is Agilent Masshunter Workstation Data Acquisition 10.0.

2.5 Method validation

For each method validation the following samples were prepared in duplicate:

- DI blanks
- DI LOD
- Matrix blanks
- Matrix LOQ
- Matrix spiked at 20% of calibration range
- Matrix spiked at 80% of calibration range

Day 0: Preparation of sample bottles

To prepare the samples mentioned above a set of 12 1 L borosilicate sampling bottles were taken from our laboratory sample bottle stores; the purpose of this was to mimic real samples to identify any contamination issues with the current process of cleaning and reusing the sample bottles. Eight bottles were each filled with 500 mL of river water measured out using a 1 L measuring cylinder that has been rinsed with acetone and river water matrix. These bottles were labelled in duplicates of the sample list shown above for matrix water; Blank, LOQ, 20% and 80%. This process is repeated for the saline method validation. Eight of the bottles were filled with 500 mL of DI water after rinsing the 1 L measuring cylinder with acetone and DI water and split, four for freshwater validation and four for saline validation. For each set these were labelled in duplicates of the DI samples shown above; DI blank, DI LOD. Spike 500 µL of each sample spike (see Section 2.5.2.4) into the corresponding labelled bottles. Swirl to mix and refrigerate overnight. The purpose of leaving overnight was to allow time for any issues the compound may have with adhering to the sampling bottle to occur, again to imitate real life samples that would be in the bottles for a period over 24 hours before extraction. The procedure outlined in Section 2.3 is then used to extract the samples.

The method validation was undertaken using a set of calibration standards, quality controls and blank samples. These samples were prepared as follows:

2.5.1 Calculations of HBCDD solutions for method validation

Calculations of the following dilutions for each solution were made using the following equation (7):

$$C_1V_1 = C_2V_2 \qquad (7)$$

Where:

C_1 = Initial concentration of solution

V_1 = Initial volume of solution

C_2 = Final concentration of solution

V_2 = Final volume of solution

Labelled standard

The stock standard for isotopically labelled HBCDD consisted of a mixture of $^{13}C_{12}$ α-HBCDD, β-HBCDD and γ-HBCDD each at 10 mg/L in toluene. The internal standard was made from a 0.05 mg/L intermediate standard solution prepared by adding approximately 5 mL of methanol to a 10 mL class A volumetric flask, spiking 50 µL of the 10 mg/L stock solution into the flask and made up to the line with methanol. However, for each matrix alternative standard solutions were made for the internal standards:

2.5.1.1 Saline matrix (2.5 µg/L internal standard solution)

Approximately 5 mL of methanol was added to a 10 mL class A volumetric flask with 500 µL spike of the 0.05 mg/L intermediate solution spiked into the flask and made up to the line with methanol.

2.5.1.2 Freshwater matrix (5.0 µg/L internal standard solution)

Approximately 5 mL of methanol was added to a 10 mL class A volumetric flask with 1000 µL spike of the 0.05 mg/L intermediate solution spiked into the flask and made up to the line with methanol.

2.5.1.3 Samples (1.25 µg/L internal standard solution)

Approximately 5 mL of methanol was added to a 10 mL class A volumetric flask with 250 µL of the 0.05 mg/L intermediate solution spiked into the flask and made up to the line with methanol.

2.5.2 Stock standards

The 100 mg/L stock standards for α-HBCDD, β-HBCDD and γ-HBCDD were also diluted into two intermediate solutions as follows prior to spiking:

2.5.2.1 100 µg/L intermediate solution

Approximately 25 mL of methanol was added to a 50 mL class A volumetric flask and 50 µL of each of the 100 mg/L stock standards were spiked into the flask and made up to the line with methanol.

2.5.2.2 15 µg/L intermediate solution

Approximately 50 mL of methanol was added to a 100 mL class A volumetric flask and 15 mL of the 100 µg/L intermediate solution was spiked into the flask and made up to the line with methanol.

2.5.2.3 Calibration spiking standards

Each calibration spike was made up using a 10 ml class A volumetric flask. Approximately 4 ml of methanol was added to each flask and spiked with the following, then made up to the line with methanol. Spiking was achieved using a 100 µL positive displacement pipette with 0.2 µL graduations, and a 1 mL positive displacement pipette. See Table 2.5 for the spiking volumes for each calibration level. To make the calibration standards, 2 mL silanised vials were spiked with 400 µL of methanol, 50 µL of the calibration spiking solution shown in Table 2.5 and 50 µL of the calibration internal standard. These were then ready for analysis.

Table 2.5: Concentrations and volumes for calibrations standard spiking solutions

Matrix	Calibrant level	Concentration (µg/L)	Volume of 15 µg/L spike (µL)
Saline	1	0.025	166.6
	2	0.075	500
	3	0.250	1666
	4	0.500	3332
	5	0.750	5000
Freshwater	1	0.050	333.2
	2	0.150	1000
	3	0.500	3332
	4	1.000	6664
	5	1.500	10000

2.5.2.4 Method validation spikes

The spikes were made up using class A volumetric flasks. The flasks were filled to approximately half the maximum volume and spiked with the following, then made up to the line with methanol (see Table 2.6 below):

Table 2.6: Concentrations and volumes for method validation spiking solutions

Matrix	Validation spike	Actual concentration (µg/L)	Volumetric flask volume (mL)	Volume of 15 µg/L spike (µL)
Saline	LOD	0.025	100	166.6
	LOQ	0.075	50	250
	20% spike	0.150	50	500
	80% spike	0.600	50	2000
Freshwater	LOD	0.050	100	333.2
	LOQ	0.150	50	500
	20% spike	0.300	50	1000
	80% spike	1.200	50	4000

These solutions were spiked at a volume of 500 µL into appropriately labelled borosilicate bottles filled with 500 mL of matrix water. The labelled standard (2.5.1.3) was spiked into the bottles at 100 µL for saline matrix and 200 µL for freshwater matrix.

2.5.3 Biota Sample Preparation

Salt extraction

1. A whole dab fish (minus the entrails) was chopped and homogenised first by a cleaver and hand blender, then by an Omni Bead Ruptor 24 Elite (see Section 5.1.1 for details). 10.0 ± 0.1 g of the homogenised fish was weighed into a 50 mL QuEChERS tube, and 2 ceramic homogenisers added. This was done for a total of 9 tubes, triplicate for each dispersive solid phase extraction (dSPE) type being tested.
2. 2.6 mL of UHP water was added to each tube and spiked with 100 µL of 160 µg/L HBCDD isomer mix. The tubes were mixed on a vortex mixer for 30 seconds.
3. 10 mL of acetonitrile (ACN) was added to each tube and then a sachet of QuEChERS salts. The tubes were vortexed again for 30 seconds.
4. The tubes were then centrifuged at 4600 rpm for 5 minutes.

Clean-up

1. 8 mL of ACN (colourless supernatant) was pipetted into a dSPE tube – triplicate tests for each of the fruit and vegetable dSPE, the fruit and vegetable with fats and waxes dSPE and the meat dSPE. These were vortexed for 30 seconds.
2. The tubes were then centrifuged, again at 4600 rpm for 5 minutes.
3. An aliquot of the top layer of ACN extract was transferred to a silanised vial for analysis.

2.6 Calculations and statistics used for method validation

The raw data obtained during method validation was entered into a statistical software application called NS30[35]. See Appendix B for the calculations and statistics used within the software.

2.6.1 Formulae used from NS30[35]

$$\% \text{ Accuracy} = (\bar{x} / \mu) \times 100$$

μ = true value

Limit of detection (LOD) = $4.65 \times s_w$

Limit of quantitation (LOQ) = $10 \times s_w$

where s_w = within-batch standard deviations

2.6.2 Instrument detection limit

The formula used to calculate the instrument detection limit (IDL) during the Agilent 6495 evaluation is as follows:

$$IDL = 2.228 \times (\%RSD/ 100) \times \text{amount measured}$$

3. LC-MS/MS METHOD DEVELOPMENT

Analyte quantitation with confidence requires a reliable and accurate measurement of the target analyte and internal standard analogues used to normalise the signal from the detector. This is often achieved through a process of method development and optimisation, followed by a final set of measures against known standards as a validation process. However, to reliably detect the analyte, the method must first show that it can measure the analyte without interference from other sample components and provide a repeatable signal abundance for representing the amount of analyte measured by the detector. Given the sensitivity required for the environmental monitoring (e.g., EQSD) of the family of isomers of HBCDD are at the pg/L level, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed using a Shimadzu 8060 LCMS.

3.1 Analyte detection

To optimize the sensitivity of the method, an initial investigation as to which ionisation approach would be best suited to monitoring HBCDD. This involved using both negative ion electrospray ionisation (ESI) and Atmospheric Pressure Chemical Ionisation (APCI) given that studies have shown that HBCDD can be detected with both these sources[17]. To tune the mass spectrometer for the target ions of each analyte, the sample loop and column were removed from the LC system to mimic a flow injection analysis, where the path of sample to the mass spectrometer was as simple as possible. Following this, a 1 mg/L sample of t-HBCDD (a bought-in mixture of the α, β and γ isomers) was injected and analysed using the relevant ionisation mode. Given the success of methanol and acetonitrile as organic mobile phases used in the analysis of HBCDD[36], methanol was used as the initial diluent solvent. To accommodate the anticipated precursor ion species determined from the monoisotopic mass, a Q3 scan was used over the mass range spanning *m/z* 550.0 – 750.0. To further optimize the sensitivity of the ionisation approach, the desolvation, spray and mobile phase flow parameters were adjusted. These parameters were evaluated over the range of operational values for triplicate measurements, with those values that provided the highest peak intensity (height) and the resolution between the α and β isomers taken as the optimum point for the parameter (see Chapter 2: Methods and Materials for optimum conditions). For example, the nebuliser gas flow rate, heating gas and drying gas of the ESI source were evaluated along with the temperatures of the desolvation line, the heating block, and the interface were optimised using the built in optimisation software of the instrument; this involved testing 60 different configurations of the 3 parameters, with the most intense peak height given at a desolvation line temperature of 200°C, a heating block temperature of 400°C and an interface temperature of 300°C (see Appendix C). Finally, the interface voltage was optimised over the range of -4 kV to -1 kV, using 5 replicates, with a value of -3 kV shown to give the largest peak height and greatest sensitivity of the target analytes. While for APCI, interface voltages were tested between -1 kV and -5 kV (1 kV increments), with -5 kV selected as the optimum voltage. For the mobile phase, flow rates were tested in triplicate between 0.4 – 1 mL/min, with increments of 0.2 mL/min and the resolution calculated. Using this approach, an optimum flow rate of 0.4 mL/min was selected for delivering the sample (see Table 3.0 and Appendix D for plot of results). The experiment was not conducted at a flow rate lower than this as APCI shows improved ionisation efficiency at higher flow rates than ESI due to a mechanism where the

mobile phase vaporises and acts as a reagent gas. The higher the flow rate, the more reagent gas molecules there are to interact and ionise the analyte[37].

Table 3.0: Calculated resolution between the α and β isomers at different flow rates with APCI source

Mobile phase flow rate (mL/min)	Resolution between α and β isomers			Resolution between β and γ isomers		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
0.4	1.97	2.14	2.11	2.71	3.00	2.82
0.6	1.91	2.09	1.87	2.54	2.55	2.37
0.8	1.75	1.71	1.95	2.38	2.29	2.43
1.0	1.62	1.66	1.45	2.24	2.19	2.09

The isomers were observed in negative ESI mode as deprotonated molecule ion species $[M-H]^-$, with the monoisotopic species at *m/z* 640.7. The *m/z* 640.7 is part of a cluster of peaks at 2 units apart and the pattern is what would be expected of a compound containing six bromines. This is because the ratio of ^{79}Br and ^{81}Br isotopes is 50.5: 49.5, so can be approximated as a 1:1 ratio, so when the likelihood of each potential isotopic variation of Br_6 is calculated, the ^{79}Br ^{81}Br combination is twice as likely to occur than ^{79}Br ^{79}Br or ^{81}Br ^{81}Br; when this is applied to the 6 bromines present in the compound, the given isotope pattern is expected.

Figure 3.0 showed two unexpected peak clusters, with predominant *m/z of* 676.7 and 703.7 respectively. The *m/z* 676.7 cluster was a chlorine adduct $[M-H+^{35}Cl]^-$ and while this was unexpected, it has been observed in other published studies of HBCDD analysis, also utilising an ESI source and in negative polarity mode.[38] the source of the chlorine adduct is unknown though it may have been an impurity in the solvents used. The *m/z* 703.7 peak cluster was likely to be $[M-2H+Na+^{35}Cl]$. The sodium adduct in this cluster may have come from glassware used to make the spike standard.

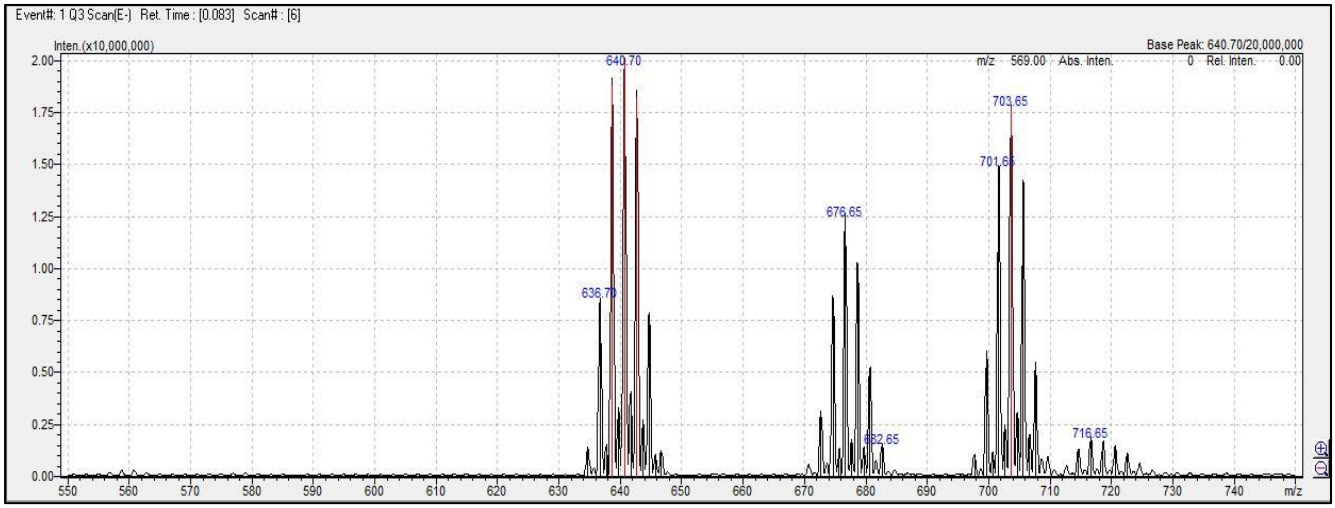


Figure 3.0: Fragmentation spectrum of HBCDD in a Q3 scan, including the cluster peaks showing at m/z 640.7, 676.7 and 703.7

When using APCI, *m/z* 640.7 and 638.7 were also observed for the three isomers however, the sensitivity was considerably lower than ESI, and required single ion monitoring (SIM) to achieve a detectable signal. Furthermore, replicate tests showed an issue with sporadic noise, giving peaks with retention times very close to the HBCDD isomers – Figure 3.1 below shows one example. Therefore, given the very low detection limits of HBCDD needed by the regulatory framework, ESI was selected as the best option ionisation to meet the demands of method validation.

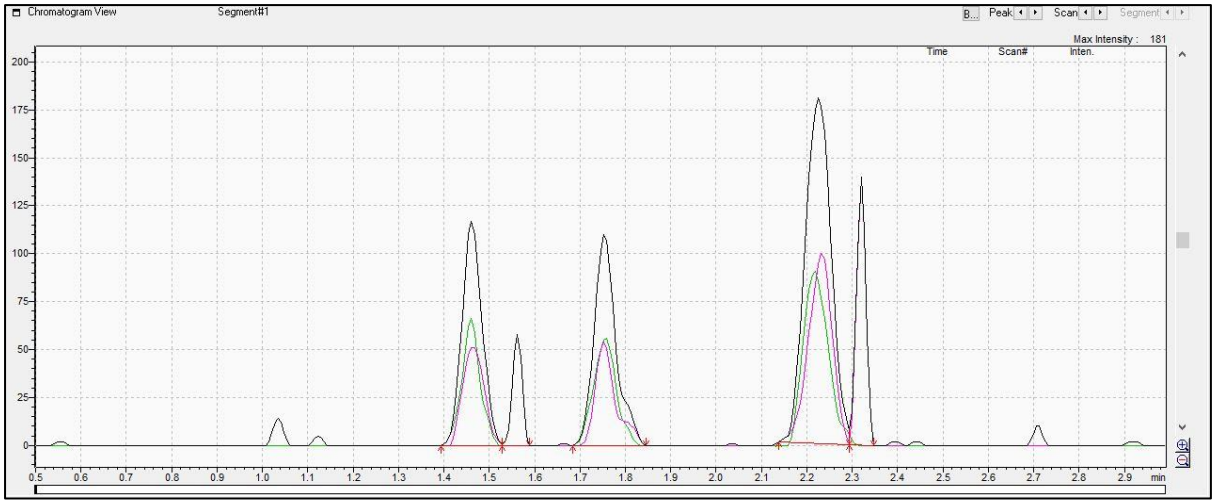


Figure 3.1: Noise surrounding the HBCDD isomers using an APCI source

3.2 Optimizing method selectivity

Selectivity is the ability of a method to determine a particular analyte in a complex matrix without interference from other ingredients of the matrix.[39] In practical terms, this can be described as the resolution or meaningful measure of separation of peaks in a chromatogram or the mass spectrum. Some factors that affect the selectivity of the analytical protocol are related to data acquisition (e.g., multiple reaction monitoring or MRM and collision energy) and the separation conditions (e.g., the LC-column used in regard to the stationary phase, bead size and pore size, the chemistry of the mobile phases, the proportions used to separate the analytes and the temperature of the column). To optimize these LC-MS parameters, standard solutions of the isomers were injected onto the system and evaluated according to the ion species observed and the peak resolution of the isomers within the chromatogram (see Chapter 2: Methods and Materials for further information).

Following analyte identification using the optimum ionisation approach (i.e., ESI), the HBCDD isomers and an isotopically labelled HBCDD were analysed as individual solutions to verify the most intense product ions for each isomer for enhanced selectivity (and sensitivity). To achieve this the 'Optimization for Method' tool within the instrumentation software was used to sequentially adjust the collision energy to generate product ions. For each

of the isomers and the isotopically labelled HBCDD it was found that the *m/z* 81.0 product ion, a ^{81}Br isotope, had the greatest peak intensity, followed by *m/z* 78.9, a ^{79}Br isotope. Given this the *m/z* 81.00 product ion was chosen as the target MRM transition for further method development and quantitative analysis.

The column selected for this separation was an Agilent Poroshell 120 EC-C18 (3.0 x 50 mm 2.7 μm) reversed-phase due to the hydrophobicity of the HBCDD isomers.[40] As such, an aqueous (A) and 50:50 methanol: acetonitrile (B) mobile phase system was evaluated along with neat acetonitrile, an IPA: acetonitrile mixture, and an ammonium acetate additive for any improvement in chromatographic peak shape. To detect changes in retention time for the isomers these were first analysed as individual solutions and then as a mixture to verify the separation of the chromatographic peaks (see Appendix E). Furthermore, to test the applicability of the chromatographic method for different concentrations, the evaluation was undertaken on a range of isomer concentrations, and the chromatographic performance measured according to peak resolution, capacity factor, column efficiency and selectivity factor. These were carried out as replicate injections of 5 μL at concentrations of 0.1 μg/L, 1 μg/L and 10 μg/L. To facilitate chromatographic separation and post elution clean-up, a gradient elution programme was used and adjusted so that the mobile phase flow was directed to waste for the first 1.5 minutes, to help prevent contamination of the analytical system from the initial surge of mobile phase moving through the LC lines. The valve was then redirected to the mass spectrometer for analysis of HBCDD where the mobile phase B started at 80% and increased to 85% over 5.5 minutes. This was increased further to 100% B for 0.5 seconds and held at this point for 2 minutes with the flow redirected to waste to flush contaminants from the column and recondition it at the starting conditions (see Table 3.1 below).

Table 3.1: Mobile phase gradient programme for analysis of HBCDD

	Time	Module	Command	Value	
1	0.01	Pumps	B.Conc	80	
2	1.50	Column Oven	CTO.RVR	1	
3	5.50	Pumps	B.Conc	85	
4	6.00	Pumps	B.Conc	100	
5	6.01	Column Oven	CTO.RVR	0	
6	8.00	Pumps	B.Conc	100	
7	8.01	Pumps	B.Conc	80	
8	12.01	Pumps	B.Conc	80	
9	12.02	Controller	Stop		
10	0.00				

Pleasingly, each isomer showed baseline separation when injected as a mixture using UHP water (mobile phase A) and 50:50 methanol: acetonitrile (mobile phase B), as represented by the resolution values in Figure 3.2 below, enabling the measurement of each isomer without interference from each other. Unfortunately, the signal-to-noise was found to be poor at the 0.1 or the 1 µg/L level for quantification purposes and so 10 µg/L was deemed most appropriate for further testing.

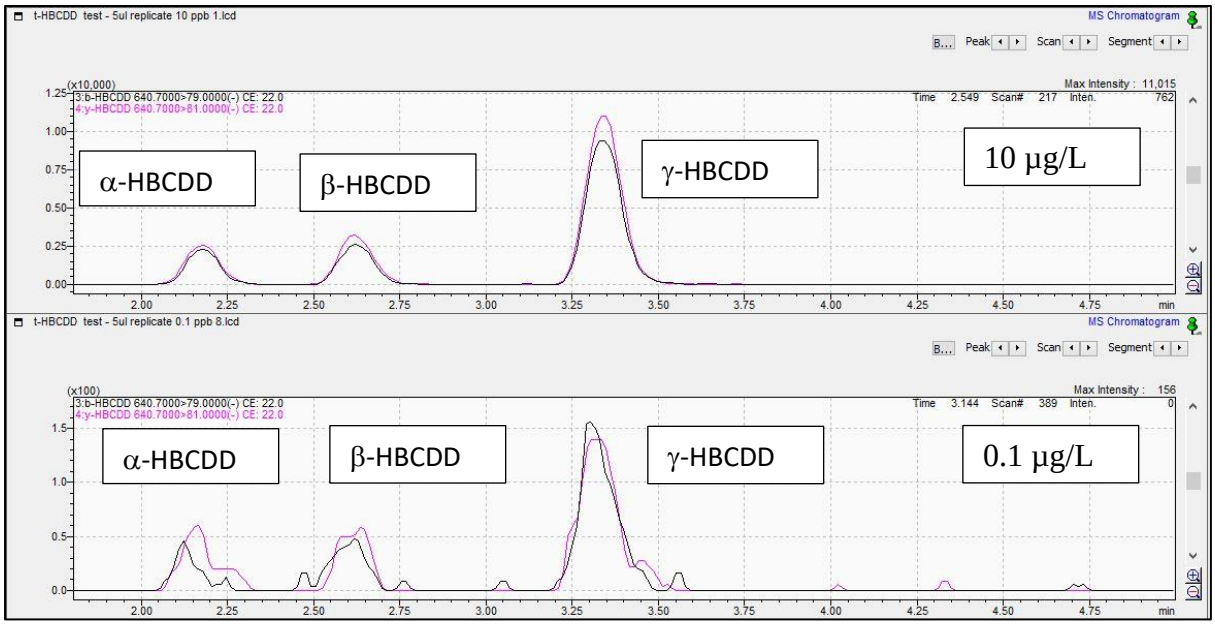


Figure 3.2: t-HBCDD at a concentration of 10 µg/L and 0.1 µg/L with a 50:50 methanol acetonitrile mix for the organic phase

Whilst, when tested with neat acetonitrile (see Figure 3.3) or an isopropanol and acetonitrile mixture as the organic phase, a substandard chromatographic separation was observed, with α-HBCDD and β-HBCDD eluting very close together (or co-eluting), and γ-HBCDD appearing a significant amount of time later with acetonitrile.

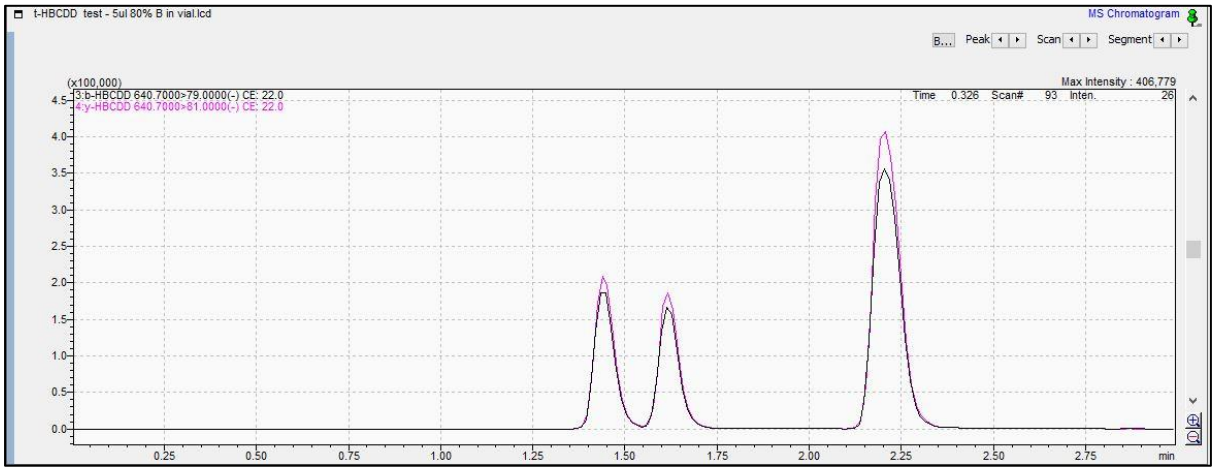


Figure 3.3: t-HBCDD using 100% acetonitrile as the organic mobile phase

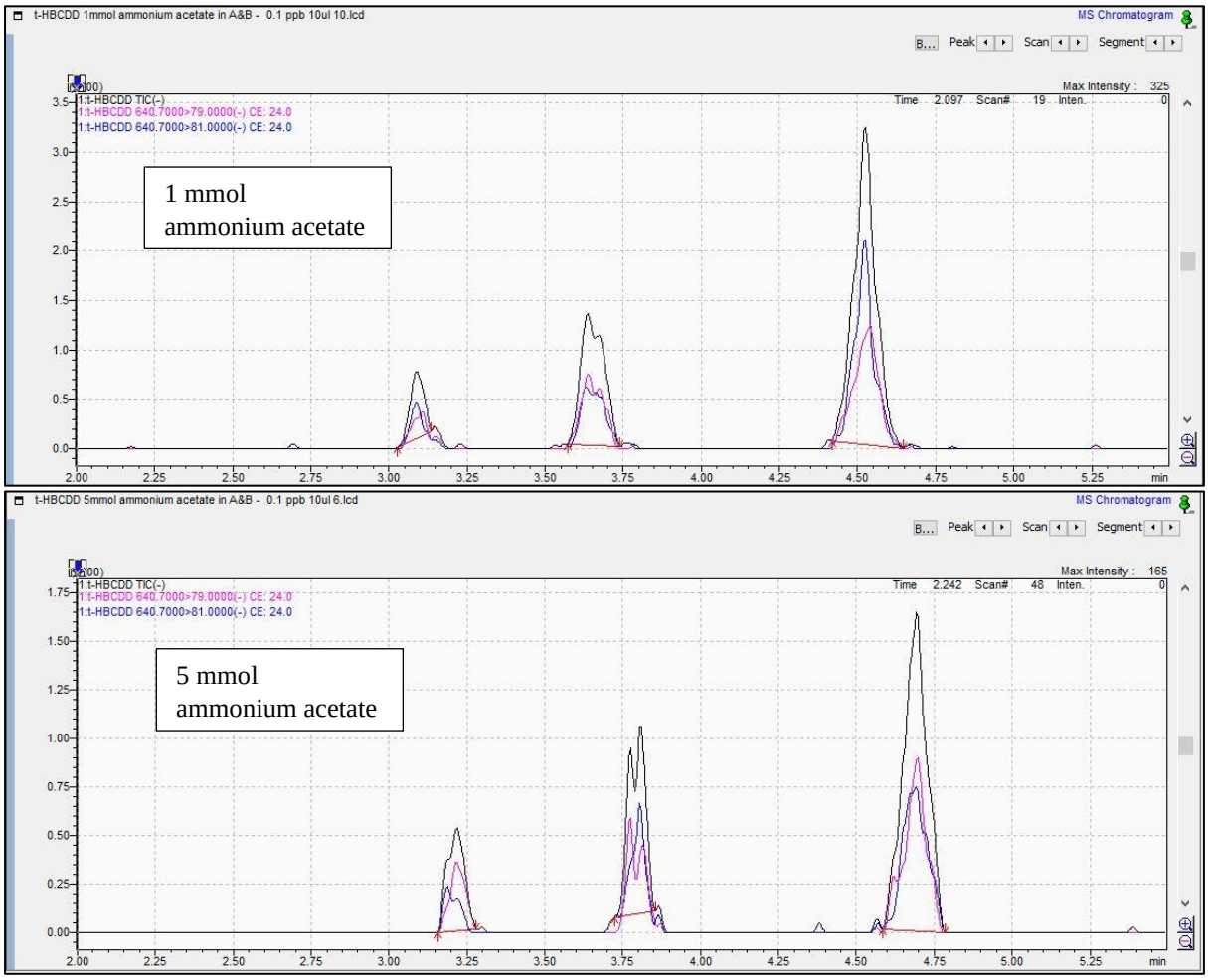
Table 3.2: Resolution of α and β – HBCDD peaks injected at 10 µg/L with a 50: 50 methanol: acetonitrile mobile phase B on the Shimadzu LC system

Mobile phase composition	**Resolution of α and β - HBCDD**
50:50 methanol: acetonitrile	1.6
acetonitrile	1.0

As Table 3.2 shows, the resolution value for 50: 50 methanol: acetonitrile is over 1.5, suggesting complete resolution of the peaks which is unsurprising as the chromatograph shows a clear baseline resolution between each peak. The resolution value for 100% acetonitrile as a

mobile phase if far lower, at 1.0. This suggests that there is a partial overlap of the two peaks and so baseline resolution has not been attained.

Finally, ammonium acetate was added to both the aqueous and organic mobile phases to make 1 mmol, 5 mmol and 10 mmol solutions. The purpose of adding ammonium acetate as a mobile phase modifier is generally to improve peak shape of basic compounds. Though HBCDD is a neutral compound, there are examples of ammonium acetate being used in other published works[41] and it was decided that this would be included in testing. This again, was tested with the 0.1 µg/L solution of t—HBCDD was injected at 10 µL. Unfortunately, ammonium acetate did little improve the peak shapes at this level at any concentration, which is to be expected as HBCDD is a neutral compound as so should remain unaffected by pH (see Figure 3.4). For high throughput HBCDD monitoring, this would be undesirable given this would extend the analysis time for a batch of samples considerably.



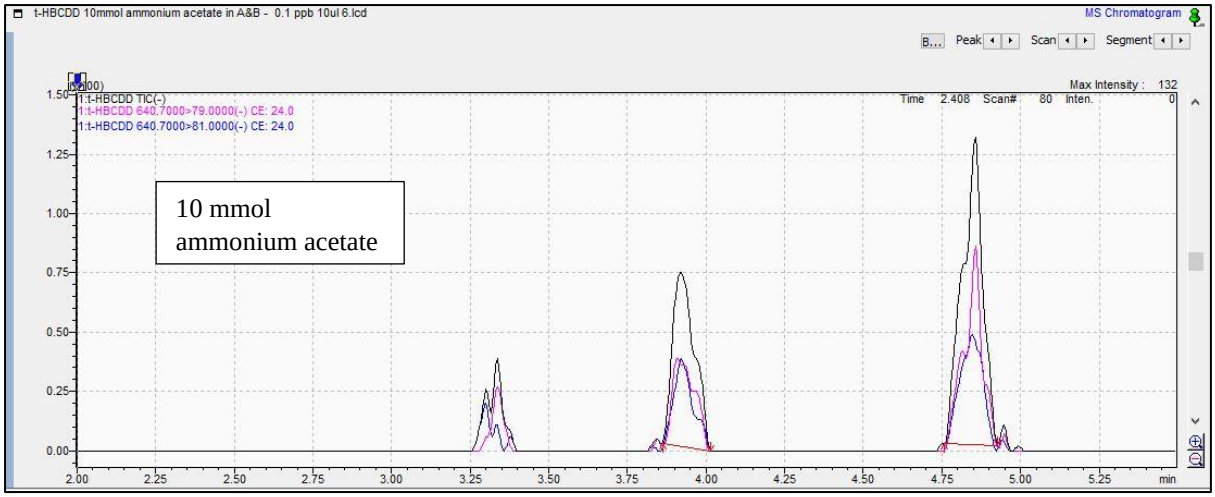


Figure 3.4: Comparison of 1, 5, and 10 mmol ammonium acetate added to both mobile phases

Given these data, it was decided that a 50:50 methanol acetonitrile mixture would be used as the organic mobile phase for further optimisation due to its improved chromatographic resolution, although it was clear that a concentration step would be required for the peaks to be reliably quantitative.

However, during prolonged operation, sporadic responses were observed for replicate samples, where the response for a replicate would appear randomly higher than others. This was attributed to a hardware design as it was apparent for some methods developed on this system but not others; for example, a similar issue was observed while conducting method validation for pesticides, though a neonicotinoid method was developed on the system without this issue. Due to the level of robustness required for this work, it was therefore decided to transfer the protocol to an alternative LC-MS system, an Agilent 1260 HPLC system interfaced with a 6495 triple quadrupole mass spectrometer.

3.3 Method transfer to alternative LC-MS

During the HBCDD method development, a new highly sensitive LC-MS system was being purchased by the laboratory and a trial analysis of HBCDD using the in-house protocol was a condition of purchase. This was to show the feasibility of application, with a lengthier

evaluation to be undertaken following installation. However, this initial evaluation trial involved the following criteria, and was undertaken by performing 11 replicate injections of a 10ul solution of HBCDD in water/methanol provided to Agilent. The solution was to be diluted until a suitable response was obtained that would allow the instrument detection limit (IDL) to be calculated.

- At least 10 data points was to be obtained across the chromatographic peak
- Only ESI was to be used
- Dilution of standards could only be made with acetonitrile, methanol, or water
- The only permitted volatile buffers permitted for testing in the mobile phase were ammonium acetate, ammonium formate, ammonium bicarbonate, ammonium fluoride, acetic acid, and formic acid
- Extracted ion chromatograms of HBCDD at or near the IDL to be provided using the following formula: IDL = 2.228 x (%RSD/100) x amount measured in femtograms. The IDL was calculated to be 3.1 femtograms on column. An extracted ion chromatogram was provided which was equivalent to 30 femtograms on-column.

  Results of the evaluation were to be provided (see Appendix F and G).

LC protocol used UHP and methanol mobile phases at a flow rate of 0.35 mL/minute, on an Agilent Poroshell 120 Phenyl-Hexyl column (2.1 x 100 mm, 2.7 µm) held at 50°C, while in place of a pump mixer an Agilent Poroshell 120 EC-C18, 3 x 50 mm, 2.7 µm column was installed. This column was selected due to the highly retentive characteristics it has due to the C18, which makes it highly suitable for aromatic compounds such as HBCDD. Again, an LC programme based on the evaluation criteria was used for separating samples (10 µL injection volume) as shown in Table 3.3.

Table 3.3: LC gradient programme created during method evaluation

Gradient	**Time**	**Mobile phase A (%)**	**Mobile Phase B (%)**	**Flow rate (mL/ min)**
	0	50	50	0.35
	1.0	50	50	0.35
	4.0	5	95	0.35
Stop Time	8.0	5	95	0.35
Post Time	3.0			

The mass spectrometer was operated in negative ionisation mode using the parameters and MRM transitions of *m/z* 640.7>78.9 and 79.9 at a collision energy of 10V (see Table 2.1 in chapter 2 Section 2.4); this was to monitor the transition as in the previous method development work, but also to enhance the sensitivity of the quantifier transition through expanding the mass extraction window and setting the product ion to the average *m/z* of the two bromine isotopes (e.g., *m/z* 79.9). Pleasingly, the IDL test showed satisfactory precision at 13.9 %RSD with a good degree of sensitivity of 3.1 and this was deemed acceptable for the method transfer (see chapter 2 Section 2.6.2 for formula to calculate IDL). The repeatability was also tested, however, in this case HBCDD was diluted with water to 30 fg on column and injected every hour over a 12-hour period. Again, good precision was observed with these measurements with an RSD of 9.54%, indicating that the analytical approach would be suitable for further method optimization.

3.3.1 Confirmation of method selectivity

The HBCDD standard provided for the Agilent evaluation test contained only one HBCDD isomer, and so one of the first tests of the more comprehensive evaluation (now completed in-house) was to verify the resolution of the three isomers using the instrument and parameters. Despite initial data indicating this platform provided a more reliable detection of HBCDD, the resulting chromatography showed poor resolution between the 2nd and 3rd eluting isomers (see Figure 3.5), suggesting that the gradient would need to be refined to separate out the peaks. The gradient time programme (see Appendix H) shows that the starting percentage of organic mobile phase is at 80% so it is likely that the HBCDD isomers are not being retained on the column long enough to allow for sufficient separation. Before doing this however, each of the isomers were run separately to confirm the identity of each chromatographic peak and indicated that the gradient would need adjusting to separate the β and γ isotopes (see Figure 3.6).

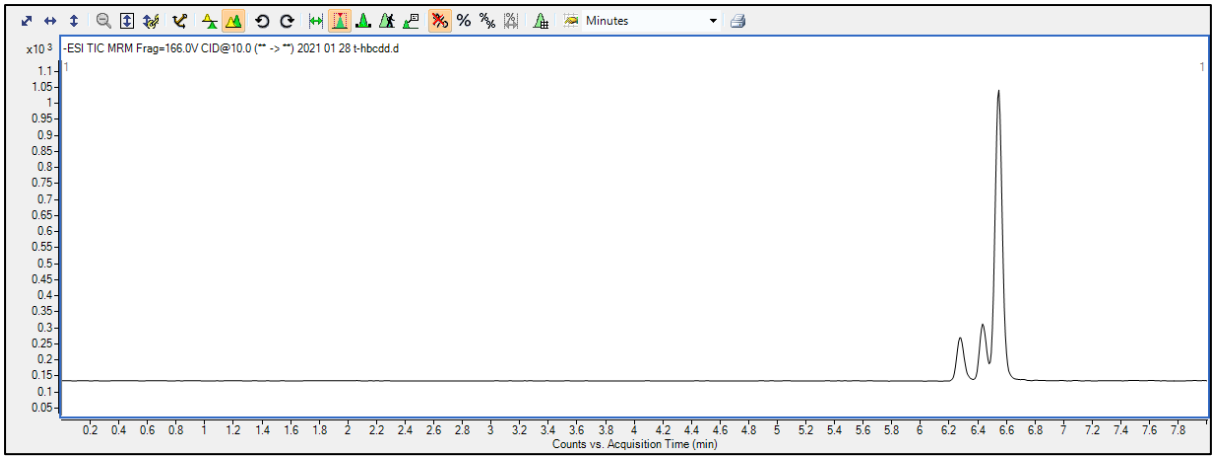


Figure 3.5: Retention times and resolution of α, β and γ – HBCDD analysed as a mix, using original evaluation method

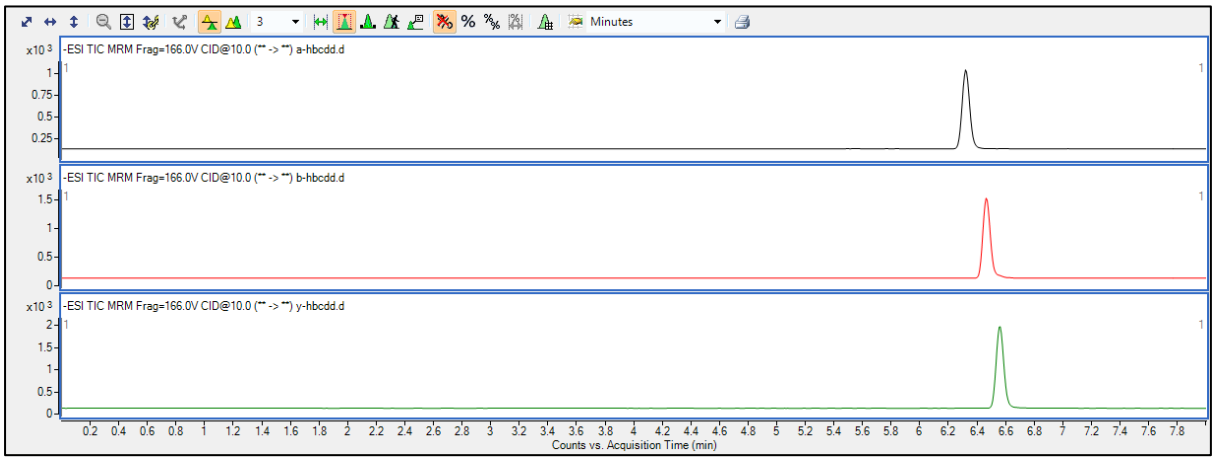


Figure 3.6: Retention times of α, β and γ – HBCDD analysed as separate standards, using original evaluation method

A brief assessment of the initial gradient developed on the Shimadzu platform using the Agilent system was conducted on $^{13}C_{12}$ HBCDD. This showed a poor chromatography for each of the isomers, giving broad, asymmetrical shouldered peaks (see Appendix I). Using the evaluation gradient as a starting point, the gradient elution profile was therefore, optimised by adjusting the % organic using a 10 µL injection of 1 µg/L of HBCDD. A total of 10 minor

alterations were made to the gradient to ensure the method included sufficient time to stabilise the pressure within the column, with the final protocol shown in the Table 3.4 below.

Table 3.4: Final version of LC gradient programme for HBCDD analysis on the Agilent system

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	50.0	50.0	0.350	500.00
1.00	50.0	50.0	---	---
5.00	15.0	85.0	---	---
8.00	10.0	90.0	---	---
10.00	5.0	95.0	---	---
11.00	5.0	95.0	---	---
12.00	50.0	50.0	---	---

To explore whether the chromatographic performance, and therefore, sensitivity could be improved further different columns were evaluated, including the Agilent Poroshell 120 EC-C18 3.0 x 50 mm 2.7 µm column connected to the InfinityLab Poroshell 120 EC-C18 3.0 X 5 mm 2.7 µm guard column used in the original method. Other columns tested included The Shimadzu Shim-pack GISS-HP C18 3 x 100 3 µm, the Agilent Zorbax Eclipse XDB-C18 4.6 x 150 mm 5 µm and the Agilent Poroshell 120 Phenyl-Hexyl 2.1 x 100 mm 2.7 µm as phenyl hexyl columns to analyse HBCDD has been researched in other published works[42] - see Figure 3.7 for comparative chromatograms of these columns.

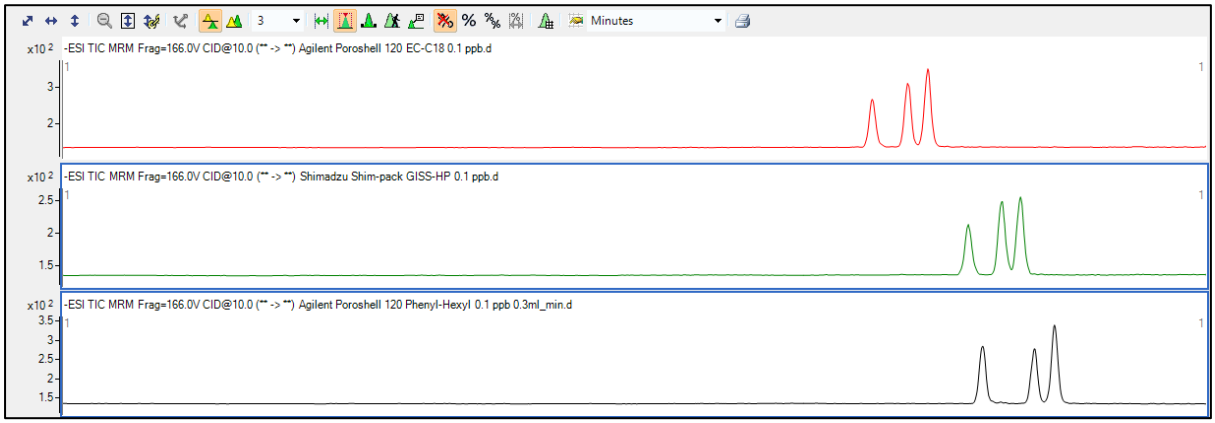


Figure 3.7: Comparison test of Agilent Poroshell 120 EC-C18, Shimadzu GISS-HP and Agilent Poroshell 120 Phenyl-Hexyl columns

The resulting chromatography shows that the Shimazu Shim-Pack column produced a lower peak intensity with slightly broader peaks compared to the other columns, so this was disregarded. There was no significant different between the Agilent Phenyl-Hexyl and the Agilent EC-C18 column chromatography (retention time difference was due to the Phenyl-Hexyl column being tested at a 0.3 mL/min flow rate as opposed to the EC-C18 column being tested at 0.35 mL/min). As neither column appeared to have an advantage over the other, the Agilent Poroshell 120 EC-C18 column was selected due to increased column availability.

The next step of the evaluation involved a Limit of Detection (LOD) test to verify the lowest concentration of HBCDD the instrument could detect given the instrument set-up. This can be determined visually using the chromatographic output, or the signal-to-noise ratio can be calculated for each sample. To achieve this, five replicates of each concentration of 0.0005, 0.005, 0.01 and 0.1 µg/L were analysed, with 0.005 µg/L showing a significant signal-to-noise value at the lowest concentration, with values of 350 for α, 448.6 for β and 533.5 for γ – HBCDD – see Figure 3.8 for comparative chromatograms of 0.0005 and 0.005 µg/L concentrations analysed.

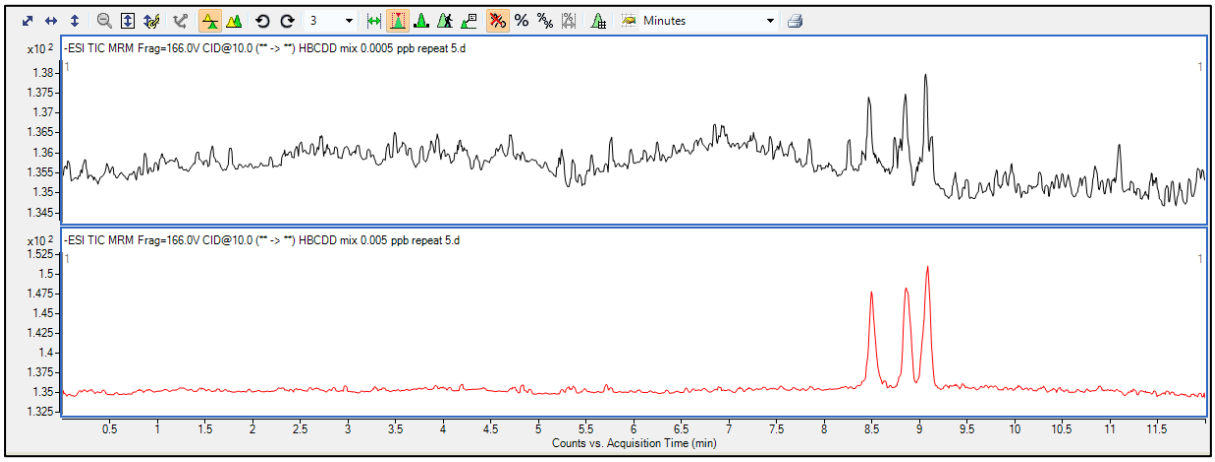


Figure 3.8: Tests of detection capability on Agilent 6495 system at 0.0005 and 0.005 µg/L concentrations of HBCDD

3.3.2 Retention time and injection repeatability

Twenty 10 µl injections were performed from a single vial containing 0.1 µg/l HBCDD to verify the repeatability of the method as it now stood (see Figure 3.9). The chromatography shows that the reinjections were very consistent, both in terms of retention times of each isomer and the response of each peak. Methanol was used for the organic mobile phase; sensitivity was better than when acetonitrile was used – although methanol mixed with water has been found to provide lower signal intensities than acetonitrile with water[43] the HBCDD isomers elute at ≥ 90% methanol, and so it is possible that due to the surface tension of neat methanol being lower than that of acetonitrile it will have a higher vaporisation efficiency in the ion source, though more investigative work would need to be done to confirm this. In addition, the cost of methanol is significantly lower so in practical terms this is beneficial for the laboratory, which will be using this method routinely.

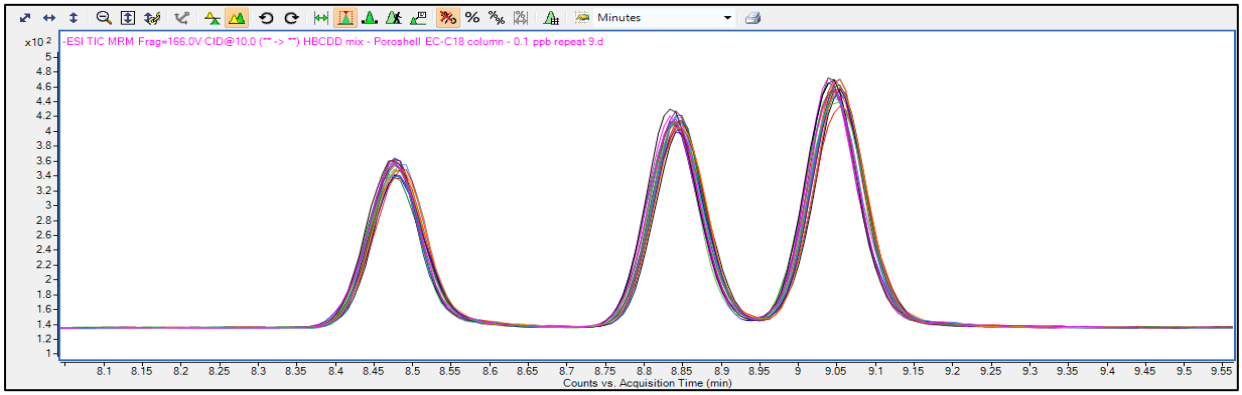


Figure 3.9: Overlaid chromatograph showing twenty replicates of 0.1 µg/L HBCDD

3.3.3 Resolution

The equation given in chapter 1 Section 1.2.1.4 was used again, this time to calculate the resolution of the β and γ – HBCDD peaks which were the closest eluting peaks on this system – see Table 3.5 below.

Table 3.5: Resolution of β and γ isomers with methanol as the organic mobile phase

Mobile phase composition	Resolution of β and γ - HBCDD
methanol	1.0

Disappointingly, resolution was calculated as below 1.5, indicating that this method does not achieve baseline resolution. Due to the repeatability of the peaks and the optimal peak shapes however, it was decided that the resolution would suffice for routine analysis. There is a risk to this approach as integration of each peak could result in reduced accuracy of each isomer; for instance, integration of the β isomer may encroach onto the γ isomer, giving a false overestimation of the result for β-HBCDD. However, with careful integration of both isomers ensuring that each are integrated discretely and given that all 3 isomers are to be summed as a total result for HBCDD, the risk was deemed as minimal.

3.4 Concluding remarks

The ESI ion source showed a significantly better sensitivity than the APCI source, and though the method developed on the Shimadzu system showed a better resolution between the HBCDD isomers the use of methanol and the EC-C18 column did give an acceptable resolution, and with the improved sensitivity and improved reliability of performance, this is a satisfactory optimisation ready for validation. The original tests for selectivity that were conducted on the Shimadzu were not repeated on the Agilent system, as a method for analysing HBCDD was provided by Agilent. If there were to be any future optimisation of the method, the effect of adding acetonitrile to the mobile phase would be assessed. Aside from this, the tests conducted on the Agilent system showed the method to be reliable and robust. These factors are paramount for a routinely used analysis in the lab and is why the Agilent system was the more suitable choice for analysis.

4. METHOD VALIDATION FOR FRESHWATER AND SALINE MATRICES

To become a routine method of quantification of HBCDD, the robustness of the sample preparation procedure through to the instrumental analysis had to be tested in its entirety and validated to meet the laboratory's UKAS requirements (see Appendix J). Of the few published methods analysing water samples for HBCDD, these did not conduct a full method validation (to the extent required for UK environmental regulation[44-48] or had not evidenced the sensitivity required for regulatory monitoring[44][47-49] with very few involving saline as a sample matrix[44]. This emphasises the importance of this work in introducing a robust analytical method capable of measuring HBCDD within the target water matrices, at the sensitivity required for regulatory monitoring. The requirements of HBCDD analysis for EQSD requires a total value for α, β and γ HBCDD isomers. The isomers were analysed separately for this validation rather than integrating the three peaks to obtain a total for two reasons; the first being that a separate integration of each peak would remove any unintentional integration of baseline and so provide a more accurate sum of the result, and because integrating the isomers separately allows for the future monitoring of bioaccumulation patterns for each isomer in water courses and interconversion between the isomers.

These validations were carried out using 11 separately prepared batches of calibration standards which were prepared in methanol and run directly on the LC-MS system, plus blanks and quality controls (QCs) that were prepared in the appropriate matrices (saline and fresh waters) to test the intra and inter-batch robustness. The resulting data was assessed using specific metrics determined by processing with the software described in Section 2.5.1 to verify whether the data passed the criteria applied, thereby confirming the robustness of the method. Given the variability of instrument response with complex matrices, internal standards (ISs) and surrogate internal standards (SISs) were used to normalise the response for quantitation and the extraction process, respectively. Having checked that the extra weight of the Carbon-13's did not affect the retention times of the isomers, there was a confidence that each isotopically labelled standard would be a more than sufficient representation of the behaviour of its native equivalent and thus would not need to undergo the sample preparation process (as is often carried out in order to mitigate the differences in behaviour between an isotopically labelled compound and a compound that will use it as a surrogate standard, but may not behave in exactly the same way). As the labelled standards for the three HBCDD isomers are presumed to behave identically to the native compounds any drop in recovery observed in the samples

(which have undergone sample preparation) compared to labelled standard in the calibration standards, both of which will have been spiked with the same equivalent concentration of labelled standard, can be attributed to loss of analyte during preparation only and the quantitative software will correct for this loss accurately. Therefore, equivalent concentrations of internal and surrogate standards were added to the calibration standards and the validation AQCs, respectively, to compensate for any ionisation (matrix) and sample preparation effects (e.g., analyte loss). The characterisation of recovery is not a laboratory requirement for validation; however, this was included within the experiment to understand how effective the method preparation procedure was for each isomer in each matrix. Each sample batch was prepared as described in chapter 2 Section 2.5.2.4 and, when ready for analysis, were run in a randomised order, to negate any potential bias that may have been introduced by running them in the same sequence for each batch. A 'system suitability' sample was also run at the beginning of each batch, as a check of the instrument performance, ensuring that the chromatographic separation was within a specific range. This consisted of HBCDD solution spiked into methanol at a concentration equivalent to the 1st calibration point of the relevant calibration method, and for the saline matrix this was anticipated to be 25 ng/L. Due to the length of the analysis run time for each batch, the saline and freshwater validation batches were run in succession and so this saline method system suitability check was used for both matrices. Once analysed, the full width at half the maximum height of the peak (FWHM), the area of the peak, the signal-to-noise ratio (S/N), and the relative retention time (RRT) of the α-HBCDD isomer were established and assessed, with the criteria in Table 4.0 used for method acceptance.

Table 4.0: System suitability criteria for HBCDD analysis in saline water (also applied as the suitability check prior to freshwater analyses).

Parameter	Accepted threshold
FWHM	≤ 0.112
Area of peak	< 265
S/N	> 60
RRT	0.999 – 1.002

4.1. Selectivity

To provide confidence that the signal being measured was representative, each batch was preceded by an initial 'no injection' where the HBCDD method was initiated, without injection

of a sample. This would ensure that any remnants of the HBCDD compound in the system would be eluted and observed in the chromatogram and used as a measure of carryover between batches. In addition to this, a 'blank' sample consisting of methanol with IS was injected after the highest calibration sample to detect carryover between injections, given the likelihood of contamination is increased with higher analyte concentrations. Using these samples, a positive test for carryover was considered if a signal indicative of HBCDD was observed in the blank injections, with a peak area of >10% of the lowest calibration point, or a signal-to-noise of >10.

Pleasingly, a positive result for carryover did not occur for any of the saline validation batches, giving confidence that the method can remove any detectable traces of HBCDD for future routine batches. However, the freshwater results did show a couple of S/N values marginally above 10, but the peak areas for these blanks were so small compared to the peak area of the first calibrant that they could confidently be disregarded as positive results. The chromatogram indicated these as spikes rather than peaks, as they did not have the required gaussian shape which indicates a true peak.

4.2 Linearity and Calibration Curve Reproducibility

To accurately quantify the concentration of HBCDD, a calibration curve that covered at least 5 non-zero points within the anticipated quantitative was prepared and analysed. Aligned with the regulatory expectations of HBCDD, a linear concentration range of 25-750 ng/ L for saline water matrix and a concentration range of 50-1500 ng/ L for freshwater matrix was used, each involving 5 different concentrations at regular intervals throughout the range with a top standard that was below signal saturation. These dynamic ranges are well in excess of past published methods[44] enabling the quantitation of a broader range of concentrations within the target samples, further emphasising the potential improvements of this method. For each method, each calibration standard was prepared independently from a single stock solution to prevent proliferation of errors in the calibration curve, given this can lead to bias that may not be immediately recognisable. The regularity between concentration points was applied to avoid data with significantly different error or bias (e.g., an outlier) exerting a leverage effect on the calibration graph[50]. The calibration standards were prepared as a spiked mixture of the three isomers and their respective isotopically labelled IS to provide a high degree of confidence that the calibration curve is a true representation of the relationship of HBCDD response and

concentration (see Section 4 for details). However, to determine any potential issues in the sample preparation stage, such as loss of sample, over-evaporation, or contamination QC samples were prepared in matrix and taken through the sample preparation – these results are discussed in Section 4.4. Once analysed, the calibration curve fit (linear and quadratic) and weighting (1/x or none) was investigated and evaluated according to the calculated concentration for each calibration point for each isomer. The average % accuracy and % Relative Standard Deviation (RSD) were calculated as per the formulae outlined in Section 2.

Pleasingly, excellent accuracy was observed across the concentration range with values spanning 96.7-102.4% regardless of the curve fit and weighting factor applied (see Table 4.1). In fact, the average % accuracy results were very similar, with little evidence to suggest which arrangement would be most appropriate. This was not surprising as the calibration standards were prepared by directly spiking the analyte into relevant solvent-based matrices and analysed without undergoing any additional sample preparation, so any variation would be due to spiking and instrument analysis only. However, the % RSD results gave a far clearer indication of which calibration curve parameters were more appropriate for method robustness. Applying no weighting to the curve produced a significant increase in the % RSD at the lowest calibration level with the highest % RSD being 28.5 for β – HBCDD, with the parameters being for a linear curve with no weighting (see Table 4.2). This is not a surprise since differences in the variation of the data points across the concentration scale can be more exaggerated at lower concentrations given the signal is more difficult to distinguish from the background noise. This heteroscedasticity of the data can be managed by applying a weighting factor, such as 1/x, to better account for this variability, and this often results in improved precision across the calibration range. This was consistent with the acquired data, with most of the lower RSD values were found with an applied weighting (1/x) factor across all 3 isomers. This was improved further by using a quadratic plotting function and, this was most significant at the lowest and highest calibration points, indicating that the weighted quadratic fit gives a higher confidence of method precision throughout this much extended calibration range versus published work[44]. Therefore, to ensure consistency in measurement for the calculation of concentrations for each proceeding batch, the calibration curve parameters were kept constant as a quadratic calibration plot function, with 1/x weighting that ignores the origin of the calibration scale.

Table 4.1: Average % accuracy of each calibration point across all 11 validation batches for the saline method, showing each calibration curve arrangement and weighting factor.

			Saline Method Average % accuracy				
Curve fit	Weighting		Cal 1 (25 ng/L)	Cal 2 (75 ng/L)	Cal 3 (250 ng/L)	Cal 4 (500 ng/L)	Cal 5 (750 ng/L)
Linear	None	α - HBCDD	96.7	98.4	101.2	100.0	99.9
Linear	1/x		99.9	99.3	101.2	99.8	99.8
Quadratic	None		99.2	98.7	100.9	99.8	100.0
Quadratic	1/x		101.1	98.4	100.7	99.8	100.0
Linear	None	β - HBCDD	102.4	100.2	99.9	99.7	100.1
Linear	1/x		100.6	99.6	99.7	99.8	100.3
Quadratic	None		98.2	100.2	100.2	99.9	100.0
Quadratic	1/x		99.6	100.5	99.9	100.0	100.0
Linear	None	γ - HBCDD	98.7	100.5	99.0	101.0	99.7
Linear	1/x		99.7	100.8	99.0	100.9	99.7
Quadratic	None		101.0	101.3	98.6	100.8	99.8
Quadratic	1/x		100.0	100.5	98.9	101.1	99.6

Table 4.2: Precision (represented by % RSD) of each calibration concentration across all 11 validation batches for the saline method, showing each calibration curve arrangement and weighting.

			Saline Method Precision (% RSD)				
Curve fit	Weighting		Cal 1 (25 ng/L)	Cal 2 (75 ng/L)	Cal 3 (250 ng/L)	Cal 4 (500 ng/L)	Cal 5 (750 ng/L)
Linear	None	α - HBCDD	21.9	4.3	4.2	4.4	1.7
Linear	1/x		5.6	4.8	4.3	4.1	2.3
Quadratic	None		18.1	3.9	5.9	2.9	0.7
Quadratic	1/x		3.5	4.4	4.0	3.9	1.3
Linear	None	β - HBCDD	28.5	7.9	5.4	3.4	1.6
Linear	1/x		5.9	4.8	5.9	3.1	2.7
Quadratic	None		19.0	5.0	4.2	1.9	0.4
Quadratic	1/x		4.5	5.6	3.1	2.8	0.9
Linear	None	γ - HBCDD	27.5	8.8	3.5	4.1	2.0
Linear	1/x		5.7	5.8	4.0	3.5	3.1
Quadratic	None		19.1	5.4	3.4	1.7	0.4
Quadratic	1/x		5.2	6.4	2.4	2.6	1.0

However, for the freshwater method the % accuracy results were slightly more varied for the lowest calibration point with data ranging from 94.5-113.9% (see Table 4.3). This was surprising given the concentration is double that in the saline method and so issues such as background noise were not expected to present as much of an issue. The largest deviations in % accuracy was observed with a quadratic curve fit where no weighting was applied, again demonstrating the importance of having a calibration curve weighted to the lower end of the graph. Whilst, for precision the % RSD results showed a similar pattern to the saline method, with weighted regression data giving a significantly improved % RSD for the lowest calibration level (see Table 4.4) with values ranging from 2.8 - 4.1. Interestingly, when comparing across the different curve fits the % RSD marginally increased for the quadratic regression data for some of the first 4 calibration points across all the isomers, although the precision improved versus linear regression for the top standard (0.4 vs. 3.1). Therefore, it was decided to use a quadratic, 1/x weighted curve fit would also be used for all subsequent analyses using the freshwater method.

Table 4.3: Average % accuracy of each calibration point across all 11 validation batches for the freshwater method, showing each calibration curve arrangement and weighting factor.

			Freshwater Method Average % accuracy				
Curve fit	Weighting		Cal 1 (50 ng/L)	Cal 2 (150 ng/L)	Cal 3 (500 ng/L)	Cal 4 (1000 ng/L)	Cal 5 (1500 ng/L)
Linear	None	α - HBCDD	94.5	96.4	99.0	103.3	98.7
Linear	1/x		101.2	98.2	99.1	103.1	98.4
Quadratic	None		113.9	99.0	96.5	102.0	99.5
Quadratic	1/x		102.8	97.5	98.0	102.8	99.0
Linear	None	β - HBCDD	101.0	99.0	97.6	102.7	99.1
Linear	1/x		101.5	99.2	97.6	102.6	99.1
Quadratic	None		111.1	100.5	96.2	102.0	99.5
Quadratic	1/x		101.5	99.2	97.5	102.7	99.1
Linear	None	γ - HBCDD	97.4	98.3	98.9	102.2	99.2
Linear	1/x		100.7	99.2	99.0	102.0	99.0
Quadratic	None		108.3	99.8	97.5	101.4	99.6
Quadratic	1/x		101.4	98.9	98.4	102.0	99.3

Table 4.4: Precision (as an % RSD) of each calibration standard across all 11 validation batches for the freshwater method, showing each calibration curve arrangement

			Freshwater Method Precision (% RSD)				
Curve fit	Weighting		Cal 1 (50 ng/L)	Cal 2 (150 ng/L)	Cal 3 (500 ng/L)	Cal 4 (1000 ng/L)	Cal 5 (1500 ng/L)
Linear	None	α - HBCDD	12.8	5.8	4.2	4.3	1.7
Linear	1/x		3.7	3.8	4.3	4.1	2.0
Quadratic	None		19.5	3.2	6.1	2.9	0.7
Quadratic	1/x		4.1	4.5	3.8	4.1	1.5
Linear	None	β - HBCDD	13.9	5.6	3.1	4.0	1.7
Linear	1/x		2.8	3.0	3.2	3.7	2.2
Quadratic	None		19.5	2.9	5.1	2.3	0.5
Quadratic	1/x		4.0	4.2	3.1	3.3	1.2
Linear	None	γ - HBCDD	16.1	5.6	2.1	4.3	1.8
Linear	1/x		1.8	2.0	2.3	3.9	2.5
Quadratic	None		17.1	2.2	4.7	2.3	0.6
Quadratic	1/x		3.5	3.5	2.7	3.3	1.2

4.3 Method Limit of Detection (LOD) and Limit of Quantification (LOQ)

One of the key elements to be proven in the validation was the sensitivity of the analysis, in relation to both the concentration at the limit of detection (LOD) and the level of quantitation (LOQ). The NS30 manual on 'Analytical Quality Control for the Water Industry'[35] describes the determination of LOD and LOQ using either a blank sample or, if complete absence of the analyte cannot be guaranteed, then a sample containing a small amount of the analyte. These are identified in Table 4.5 as experimental spike LOD, and experimental spike LOQ. Therefore, in compliance with the safety requirements of environmental monitoring, our study involved analysing a sample of ultra-high purity (UHP) water spiked with HBCDD at a concentration of x10 less than the required EQSD targets to determine the LOD (EQSDs = 0.8 ng/L for saline and 1.6 ng/L for freshwater), so LOD spikes were 25 pg/L for saline and 50 pg/L for freshwater. These results were then compared with the NS30 LOD and NS30 LOQ that were derived from applying the equations associated with NS30 (see Section 2.6.1) to the standard deviation of the DI LOD spikes.

Pleasingly, the α and γ – HBCDD LOD results showed reasonable precision with values below the 15% RSD acceptance criteria (14.3 and 11.0 % respectively) at pg/L concentrations, while β -HBCDD was slightly higher at 17.4%. However, given this concentration was x10

lower than the intended limit of quantification, and LOD was not used to determine the suitability of quantifying the eventual amounts in unknown samples, it was not considered a significant issue. Interestingly, this quantitative calculation of the LOD using a spiked standard showed far higher LODs across all isomers than the NS30 determined LOD (see Table 4.5 below) with values approximately double for α and β - HBCDD and nearly 3x higher for γ – HBCDD. This suggests that the LC-MS can detect lower HBCDD concentrations than that of the samples spiked at an estimated LOD level and gives confidence that should the EQSD requirements for HBCDD be lowered in future, this LC-MS instrumentation has the potential to detect it at lower levels. Furthermore, the greatest potential in reducing the LOD appears to be for the γ – HBCDD with sub-10 pg/L LODs suggested by the NS30 calculation. The mean value of the actual concentration of the experimental spike LOD samples was also found to be very close to the nominal value.

As anticipated, the precision of the calculated amounts improved at the higher concentrations used for the LOQ, with % RSDs for the saline LOQ spike of:

α – HBCDD = 12.1 %
β – HBCDD = 12.2 %
γ – HBCDD = 10.0 %

Again, these are desirable values given they all fall below the laboratory's % RSD target of 15%, giving confidence in the precision of the LOQ results over multiple batches and the robustness of the saline method. Furthermore, these LOQs are significantly improved on what little published data exists, where previous values have been reported at high pg/L levels (0.5 ng/L)[44]. As with the LOD, the NS30 calculated LOQ for α – HBCDD was found to be higher than the spiked value obtained from the LOQ spike whereas the β and γ – HBCDD isomers were determined to be below the experimental value. However, as the LOQ spike was already significantly lower than the EQSD target, the LOQ value was kept at a concentration of 75 pg/L, and this remains an improved method performance than currently published work.

Table 4.5: Within-batch mean result and standard deviation for the LOD spikes and LOQ spikes, the NS30 determined LOD and LOQ for each isomer within saline matrix. Results are from 11 validation batches. s_w = within-batch standard deviation for determined concentration (e.g., either LOD or LOQ)

	Nominal concentration (pg/L)	α-HBCDD (pg/L)	β-HBCDD (pg/L)	γ-HBCDD (pg/L)
Experimental spike LOD	25	26	24.9	26.3
Mean LOD s_w		2.67	2.60	1.91
NS30 LOD		12.42	12.09	8.88
Experimental spike LOQ	75	77.4	76.0	76.9
Mean LOQ s_w		8.32	4.69	5.19
NS30 LOQ		83.2	46.9	51.9

Unlike the saline matrix, data for the freshwater LOD spike showed acceptable precision for all isomers with values below the 15 % RSD target (α – HBCDD = 11.3%, β – HBCDD = 9.2%, γ – HBCDD = 4.4%), giving confidence that the freshwater method has good precision at this level. Again, the NS30 calculated LODs were determined to be significantly lower than the spiked sample values across all 3 isomers (see Table 4.6), again demonstrating that the LC-MS has the potential to detect HBCDD at lower concentrations than the given LOD sample spike, accounting for future adjustments if needed. The spiked LOD results also showed very similar values across the three isomers with a mean value of the calculated concentration very close to the nominal value. Similarly to the LOD, the precision for the experimental LOQ was acceptable for all 3 isomers with values of 9.05 %, 7.02 % and 9.42 % for α –, β –, and γ – HBCDD, respectively, providing confidence that the method is robust for the freshwater matrix at a pg/L level. This was on par with the few select publications in which pg/L method detection limits (10-30 pg/L at S/N = 3) were reported for HBCDD in river water[45] and deionised water[46]. However, these methodologies did not appear to involve a full method validation, adding uncertainty over the reliability of the protocol for regulatory monitoring. Full method validation is a requirement for regulatory environmental monitoring, to ensure the robustness of the method. Again, the spiked LOQ values were higher than those calculated via NS30, showing promise that samples quantitated at the LOQ level will be reliable, and that the LOQ could be reduced if the freshwater method requirements change in the future.

Table 4.6: Within-batch mean result and standard deviation for the LOD spikes and LOQ spikes, the NS30 calculated LOD and LOQ for each isomer within freshwater matrix. Results are from 11 validation batches. s_w = within-batch standard deviation for determined concentration (e.g., either LOD or LOQ).

	Nominal concentration (pg/L)	α-HBCDD (pg/L)	β-HBCDD (pg/L)	γ-HBCDD (pg/L)
Experimental spike LOD	50	51.9	50.0	52.3
Mean LOD s_w		2.4	2.9	2.1
NS30 LOD		11.14	13.51	9.90
Experimental spike LOQ	150	151.6	148.1	151.2
Mean LOQ s_w		12.27	8.89	8.25
NS30 LOQ		122.68	88.85	82.54

4.4 Accuracy and precision

A necessary part of developing a quantitative method is to prove it to be both accurate and precise in determining concentration. This was assessed within the validation batches by preparing extracts from duplicate matrix samples spiked at 20% and 80% of the calibration range (see Section 2.3 for details on sample preparation) and determining their concentration. These test concentrations were chosen to supplement the LOQ samples in providing a more complete coverage of the quantitative accuracy and precision for HBCDD throughout the calibration range. In addition, to the HBCDD analytes, a SIS was spiked before extraction to account for analyte loss during the disk SPE sample preparation.

The results in Table 4.7 below show that the precision for both the 20% and 80% spikes for each isomer fall far below the target value of <15% RSD, giving acceptable performance and confidence that the method provides reproducible quantitative results above the LOQ, and across the concentration range and the number of batches tested. Furthermore, the % accuracy of the results were within the desired range of 80-120%, with quantitative determinations within 98.3 – 102.7 %accuracy. Again, this falls well within the laboratory's required target range, with the 80% spike exhibiting the optimum precision for all isomers as expected given its improved S/N. The α – HBCDD isomer typically showed the best % accuracy for both concentrations, which again, wasn't surprising given this peak was fully resolved and so the integration of the peak is likely to be more consistent across all the validation batches.

Table 4.7: Summary of the saline method validation accuracy (%) and precision (% RSD) results for quantifying samples of concentration within 20% and 80% of the calibration range

		α - HBCDD		β - HBCDD		γ - HBCDD	
Matrix	Nominal conc. (pg/L)	%RSD	% Accuracy	%RSD	% Accuracy	%RSD	% Accuracy
Saline water	150 (20% of calibration range	10.3	100	12.6	102	7.57	99.3
	600 (80% of calibration range)	8.26	99.5	7.27	98.3	6.98	102.7

The freshwater method showed excellent precision across the concentrations tested, with values significantly lower than 15 %RSD for all 3 isomers (see Table 4.8). Largely similar values were also observed between the different concentrations and the isomers, giving confidence that the quantitative method is reliable for each analyte tested across the calibration range and is reproducible across multiple batches. Again, the % accuracy across all the isomers was acceptable, with values ranging from 97.5% – 99.3 %, showing a high level of accuracy associated with the method. Similar values in accuracy were observed between the isomers and concentrations tested, suggesting that this method would perform effectively with acceptable bias in quantifying HBCDD.

Table 4.8: Summary of the freshwater method validation accuracy (%) and precision (% RSD) results for quantifying samples of concentration within 20% and 80% of the calibration range

		α - HBCDD		β - HBCDD		γ - HBCDD	
Matrix	Nominal conc. (pg/L)	%RSD	% Accuracy	%RSD	% Accuracy	%RSD	% Accuracy
Freshwater	300 (20% of calibration range	5.32	97.7	6.45	99.3	6.91	99.0
	1200 (80% of calibration range)	8.06	98.3	4.64	97.5	6.04	98.3

As discussed earlier, surrogate internal standards (SIS) were used throughout the analysis; each HBCDD isomer had an individual isotopically labelled standard that was spiked into each sample before undergoing the samples preparation procedure. The equivalent final concentration of the SIS was spiked into the calibration standards, which did not undergo sample preparation, so loss of SIS during sample preparation could be observed. It was not a requisite metric to characterise SIS recovery within this validation protocol, however this was explored for saline and freshwater to understand the impact on sensitivity from the extraction method and if this would require further improvement (or an alternative method) for more complex matrices such as biota. Unsurprisingly, the recovery of the SIS did decrease when analysing the freshwater and saline samples in comparison to deionised water matrix with values typically ranging from approximately 33-41% for freshwater 34-41% for saline samples (See Appendix K). For freshwater samples, methods in previously published papers that involved significantly improved recoveries (e.g., >75%) did appear to involve more lengthy sample preparation protocols[48][49]. Furthermore, the data acquired for saline matrix did appear lower than that obtained for a previous study[44] however, this study appears to have used an alternative SPE disk, a reversed-phase C18 disk, and this may have resulted in the improved recovery for this sample. Therefore, despite these methods showing acceptable quantitative accuracy and precision from matrix, this data suggests that further development may well be required for more complex samples, where the sensitivity limits may be more challenging to achieve.

4.5 Applicability to environmental samples: freshwater

Once validated, the freshwater method was applied to a pilot set of freshwater samples (n=9) to measure the presence of HBCDD. Grab samples of 1 L were taken from selected local rivers as part of a routine sampling campaign, prepared and analysed alongside a set of calibration standards to quantify any HBCDD present and analytical quality control (AQC) standards as a check of the quantitation. The AQC standards consisted of UHP water spiked with a known concentration of the analyte of interest and surrogate standard; this underwent sample preparation and extraction alongside the samples to provide a comparative sample to show that the procedure is working correctly. This was undertaken using the method described in Chapter 2 Section 2.3 and processed using regression statistics. Unfortunately, a spiking error occurred whilst preparing the AQCs resulting in the concentration of the AQC to be

significantly beyond the concentration of the top calibration level; this was only realised during the analysis. From an internal quality perspective these AQC samples were no longer suitable for use and a new batch of samples would need to be prepared before reporting the results, however, given the calibration standards passed the acceptance criteria, and a linear relationship could be established, these were used to provide a quantitative result for the purpose of this project (see Figure 4.0).

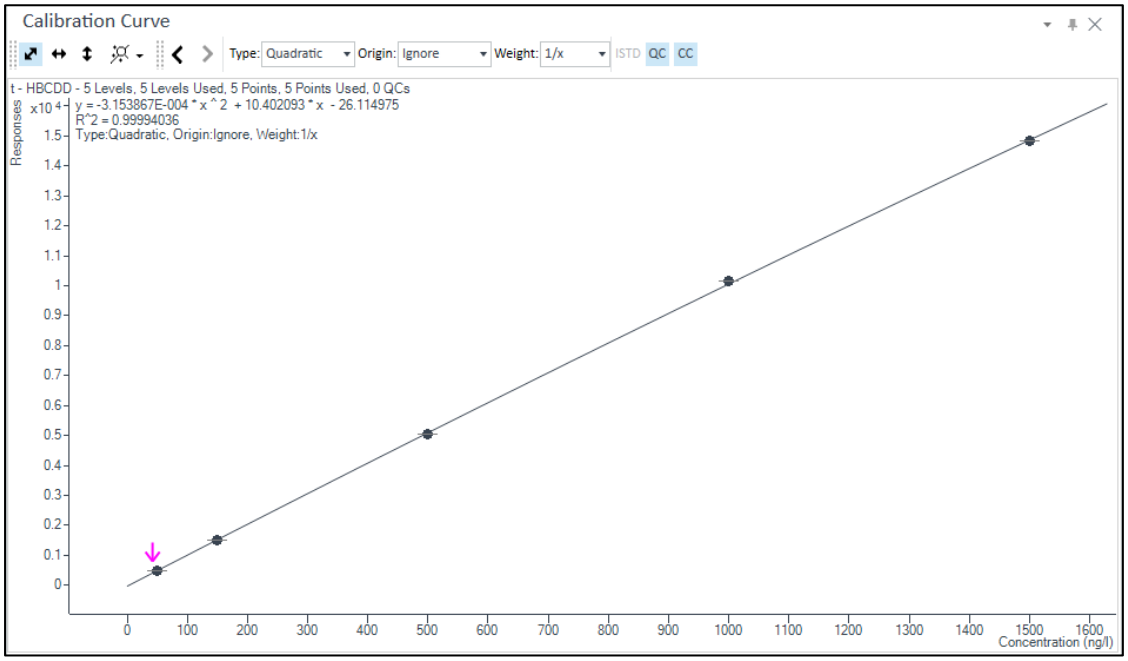


Figure 4.0: Plot of quadratic function and 1/x weighting obtained for the pilot screening experiment involving n=9 river water samples.

All three HBCDD isomers were observed above the LOD (with a S/N >3) in one of samples analysed, with isomer α showed the highest response (see Figure 4.1). When using the calibration relationship, the highest estimated concentration was determined to be ~0.49 ng/L, which exceeds the LOQ value of 0.15 ng/L for freshwater (see Table 4.9).This was not expected given other freshwater studies have shown a higher prevalence for the γ isomer. However, studies have shown that isomeric species can transform from one isomer to another; for the γ isomer of HBCDD, this is thought to occur following the exposure to light[51] and could explain the higher than anticipated levels of the α isomer. It is possible that this may have occurred during the sampling of the freshwater matrix, however this takes places in a relatively short

space of time and during transportation of the matrix bottles they are enclosed in a plastic crate without light exposure. Another possibility is that the HBCDD found has been present in the rivers for some time and had enough exposure to light in that time for isomeric conversion to take place.

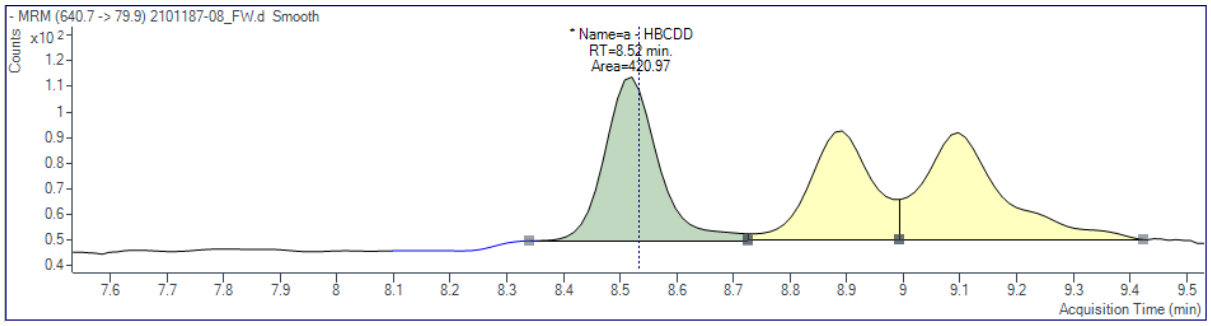


Figure 4.1: Chromatogram showing the HBCDD isomers within a river water sample.

Table 4.9: Summary of the quantitative batch for the river water samples, including S/N, peak area, and a final concentration for the calibration standards, blanks and the study samples for each isomer.

Sample		a - HBCDD Results			b - HBCDD Results			y - HBCDD Results		
Data File	Acq. Date-Time	S/N	Area	Final Conc.	S/N	Area	Final Conc.	S/N	Area	Final Conc.
No Injection.d	14/09/2021 10:27	1.32	6.95	0.1379	1.19	5.24	4.0284	1.25	6.94	1.3117
Cal 1_FW.d	14/09/2021 11:58	773.57	317.63	0.0505	1009.92	387.89	0.0510	1265.04	497.88	0.0497
Cal 2_FW.d	14/09/2021 12:13	503.67	881.85	0.1467	726.54	1228.43	0.1465	470.29	1515.05	0.1506
Cal 3_FW.d	14/09/2021 12:29	1499.29	2925.53	0.5119	2162.78	4096.36	0.4986	2456.97	5059.40	0.5040
Cal 4_FW.d	14/09/2021 12:44	4963.09	5870.70	0.9831	6971.27	8107.87	1.0102	4608.04	10152.95	0.9910
Cal 5_FW.d	14/09/2021 13:00	6097.00	8664.08	1.5078	8588.06	12021.62	1.4937	5281.10	14819.11	1.5047
Cal blank_FW.d	14/09/2021 13:15	6.22	4.81	0.0034	8.89	8.35	0.0087	26.90	16.18	0.0066
2101188-06_FW.d	14/09/2021 13:31	12.53	156.73	0.1247		0.00	0.0000		0.00	0.0000
2101244-01_FW.d	14/09/2021 13:46	16.18	145.74	0.1261	7.77	64.67	0.0362	7.67	97.96	0.0456
2101425-02_FW.d	14/09/2021 14:02	11.32	144.55	0.0855	4.69	44.16	0.0232	1.89	47.62	0.0202
Prep blank_FW.d	14/09/2021 14:33	6.46	16.98	0.0088	3.72	9.94	0.0103	2.77	13.78	0.0079
2101167-04_FW.d	14/09/2021 14:48	7.35	20.15	0.0123	14.29	29.44	0.0160	7.03	139.36	0.0411
2101163-02_FW.d	14/09/2021 15:04	1.37	14.36	0.0145		0.00	0.0000	1.84	69.61	0.0325
2101187-08_FW.d	14/09/2021 15:19	30.03	479.15	0.4906	19.27	332.30	0.1712	20.95	410.10	0.1838
2101327-01_FW.d	14/09/2021 15:50	5.56	26.95	0.0169		0.00	0.0000	3.86	128.76	0.0439
2101365-01_FW.d	14/09/2021 16:06	5.68	48.67	0.0364	5.18	40.27	0.0211	2.97	118.96	0.0417
2101425-01_FW.d	14/09/2021 16:21	7.79	41.33	0.0299	3.32	15.21	0.0133	3.39	92.62	0.0362

This work, therefore, provides preliminary data of local contamination of environmental samples, and in particular river water, that was previously unavailable. The data clearly indicates a need for further study across a wider selection of rivers and sample types (e.g.,

saline), to map the expected isomeric ratios for the environmental catchments tested, for a better understanding of the extent and reach of this pollution.

4.6 Concluding remarks

This work has shown highly robust measurement methods, that successfully pass a lengthy validation process recognised by UK regulation, are possible for HBCDD. These methods have been developed for both saline (including saline) and freshwater matrices and have shown a good degree of accuracy and precision in quantifying the HBCDD isomers, at the level of sensitivity required for the regulatory monitoring. Pleasingly, this is comparable to or much improved on published work despite lower recoveries for the surrogate internal standard than these studies. For example, recoveries where the approximate recoveries are 60% in DI for both methods, 40% for saline matrix and 37.5% for freshwater matrix, this applied across the 3 isomers, and show that further gains in sensitivity may be achieved with this protocol through potential sample preparation adjustments (e.g., an alternative SPE disk). Once validated, these methods are ready for application to complex matrix monitoring and have been applied as part of a pilot study for freshwater samples. Samples were taken from a selection of rivers as part of routine monitoring campaigns and a positive result was observed, exhibiting evidence of HBCDD contamination. These samples showed levels up to ~0.49 ng/L and this exceeded the LOQ set for freshwater. This evidence further justifies the development of these measurement protocols for HBCDD, along with other environmental matrices (e.g., biota), and a more prolonged sampling campaign to better understand the extent and reach of pollution.

5. SAMPLE PREPARATION METHOD DEVELOPMENT FOR THE BIOTA MATRIX

In addition to the aqueous matrices described in the previous chapters, the EQSD also requires the analysis of HBCDD concentrations in biota, specifying that in this instance, ‘biota’ refers to fish. A lead specialist advisor for inland and coastal waters was consulted on the species of fish that would be selected and it was decided that dab would be an appropriate

choice due to its prevalence in both local waters and other UK seas, allowing future comparisons to be made across the UK. Given biota is a highly complex sample, known to contain a high number of lipophilic substances that can suppress ionisation when using ESI, a sample preparation or extraction method would be required to measure any HBCDD content within this matrix. However, no existing method was available within NRWAS and this would require development given published methods had commonly used a laborious Soxhlet extraction, unsuitable for this work due to the length of time it would take to prepare the samples. [14] A more recently developed sample preparation for a range of non-polar and polar compounds is the QuEChERS protocol. This uses a two-stage approach to extracting complex matrices, originally pesticides from foodstuffs, and has shown potential for an increasing breadth of sample matrices[52-68] and target substances. Therefore, given the application to environmental, clinical and food matrices, and the polarity compatibility of HBCDD with the initial extraction solvent (ACN), QuEChERS was investigated a potential extraction method.

To understand the sensitivity required from the sample preparation (and analytical method), the target reporting value, LOD and LOQ must first be calculated. For example, the given EQSD value for HBCDD in biota is 167 µg/kg. This was divided by 3 and then rounded down to 55 µg/kg to provide a target total HBCDD sensitivity as HBCDD will be analysed as 3 separate isomers; these will then be summed to give the total value for reporting. To establish the target LOD sensitivity (at a value x10 lower than the EQS) a concentration of 5.5 µg/kg will be required. Similarly, for the LOQ, as a value x3 lower than the EQS, a nominal concentration of 18 µg/kg would be required. It was anticipated that these sensitivities would be quite challenging to meet with the increased complexity of the biota matrix, and a greater likelihood of the co-extraction of substances during the sample preparation and chromatographic co-elution would be expected with this analysis. Therefore, adjustments of the mobile phase composition used with the analytical method were explored to help provide as much chromatographic resolution between the isomers as possible. To do this, the instrument parameters were transferred from the aqueous HBCDD method and tested using 100% acetonitrile (ACN) as the organic solvent in the mobile phase for each HBCDD isomer separately as a 10 µg/L solution, to verify whether this would result in a change in the elution order. Pleasingly, the elution order of the isomers remained the same as the 100% methanol (MeOH) mobile phase (e.g., the aqueous methods), so further tests using the isomer mixture could be used with confidence. However, to establish the optimum mobile phase composition, a range of ratios from 10% MeOH/ 90% ACN to 100% MeOH were tested by injecting a 0.1

µg/L mixture of the HBCDD isomers. As the LOQ target is 18 µg/kg (as mentioned above) and taking into account the sample weight needed per the sample preparation method is 10 g, the equivalent concentration of HBCDD at LOQ target level in this volume of sample would be 0.18 µg/kg; this was rounded down to 0.1 µg/kg to simplify the procedure. Unsurprisingly, at 100% MeOH the β and γ – HBCDD peaks begin to co-elute as observed with the aqueous method. However, different ratios of acetonitrile (ACN) and methanol (MeOH) were tested, and it was observed that the higher the percentage of MeOH in the mixture the greater the response of the isomers; this was facilitated by an increase in the retention times of the peaks, providing greater opportunity for the α and β – HBCDD peaks to be fully resolved, and a reduction in background noise at the 70:30 MeOH: ACN ratio. The ratio of the organic solvent also appeared to change the ratio of each isomer, with the most prominent difference being observed with γ-HBCDD, showing a significant increase in response with the proportion of MeOH. Overall, the ratio of 70:30 MeOH: ACN provided the highest chromatographic resolution whilst achieving a consistent isomer intensity ratio (as each isomer was analysed at the same concentration), and was therefore, selected as the organic mobile phase (see Appendix L).

5.1 Evaluation of HBCDD extraction by QuEChERS

The QuEChERS initial extraction step used in the preliminary testing was the original, unbuffered method. This contained magnesium sulphate ($MgSO_4$) and salt (NaCl), without the buffering agents included in the modified method versions, namely the European Committee for Standardisation (CEN) method and the Association of Analytical Communities (AOAC) method, developed for pH-dependent pesticides. This is the method in its simplest form and was the logical choice for extraction of HBCDD given this is a non-polar compound that should not be sensitive to pH changes. However, given the complexity and solidity of the matrix, several modifications were made to ensure sufficient homogeneity and extraction of the material could take place.

5.1.1 Sample homogenisation: Standard procedure vs Omni Bead Ruptor Elite

Homogenisation using chopping, grinding or bead milling is a well-accepted technique to improve sample consistency and release of substances previously trapped within tissue.[69]

The current procedure at the laboratory for homogenisation of biota materials is to manually chop up the matrix using a cleaver, use a hand blender to achieve a fine paste and then finally mix it all together to attain homogenisation. Though this has worked sufficiently for previous biota matrix, it is difficult to achieve with dab fish due to the bones. A pair of kitchen shears were purchased to test whether the initial chopping of the fish could be achieved with more ease/ less splash of the biota. It was possible, but it did take time (approximately 30 minutes) to complete a single fish. Other alternatives are being considered, such as cryo-freezing the fish before homogenisation.

After the initial chopping of the biota, the efficiency of the in-house homogenisation technique was compared to dedicated homogenisation equipment for this matrix. The Omni Bead Ruptor Elite was used for initial tests to see whether there was a visual improvement of the homogenate and to compare the time taken for both methods. This involved weighing out the pre-blended biota into specifically designed Omni tubes, containing 2.8mm ceramic beads. Initial tests were done using 30 mL tubes, though after discussions with the manufacturers it was recommended that the biota mass in the tube would not exceed 2/3 of the tube capacity, and so it was decided that using 50 mL tubes would be more efficient to homogenise more biota with less matrix loss that can result from biota sticking to the ceramic beads.

The Omni Bead Ruptor Elite, which Swansea University allowed use of for the purpose of this method development, has pre-designed programmes for homogenisation or can be modified as needed. The initial tests were conducted using a pre-designed programme which consisted of a speed of 5 m/s, 30 second cycle time, 30 second pause time, with a total of 3 cycles. Pleasingly, this programme resulted in a visible improvement of the homogenate, which now appeared as a thick paste without lumps (see Figure 5.0 below). To test this further, the homogenate was left to cool for 5 minutes and then put back through the programmed cycle. Again, the homogenate was visibly smoother than the previous round. At this stage, the ceramic beads were completely dispersed within the matrix, and so the tubes were placed into a refrigerated centrifuge at 2000 rpm for 3 minutes to pull the beads to the bottom of the tube, allowing for easier removal of the matrix.

Figure 5.0: A visual comparison of the Omni homogenisation technique (left) and a standard hand blender technique (right)

To investigate whether shorter cycles with more pauses would result in faster homogenisation the programme was adjusted to a 10 second cycle, with 10 seconds dwell between each cycle, for a total of 9 cycles so that the overall homogenisation time would equal the original programme. However, this programme caused the tubes to heat up significantly, this was undesirable as an aim of the method development was to avoid thermal degradation of the sample components within the matrix. To counter this, it was decided that in future tests, the effect of placing the tubes in cold water to bring the temperature down as quickly was possible would be investigated.

5.1.2 Sample hydration

A minimum percentage of water present in the sample (e.g., 80%) is known to facilitate the performance of the extraction for polar compounds. As HBCDD is non-polar, the role of hydrogen bond disruption between the biota and HBCDD that the water would enable does not factor here, however the water will increase the fluidity of the sample and the mobilisation of the extraction salts. Fish is not specified as a matrix for use with QuEChERS, but for 10 g of sample matrix that contains 25-80% water[70], a volume of water equivalent to the sample

weight (10 g) minus the amount of water found in 10 g of the matrix, should be added. In the absence of a direct reference to fish as a matrix type, the required water volume was calculated by determining the moisture content through comparing equivalent amounts of wet sub-samples of dab weighed into two polystyrene weighing boats, before and after freeze drying (see Table 5.0).

Table 5.0: A summary of calculations for moisture content of dab fish

	Duplicated sample test	
Duplicated samples	**A**	**B**
Boat weight (g)	1.82	1.66
Wet weight of dab + boat (g)	9.04	8.41
Dry weight of dab + boat (g)	2.31	2.19
Dry weight (%)	26	26
Moisture content (%)	74	74

Using this as an approximation across the dab fish tested, this moisture content % was applied to all future biota matrix consisting of dab, and the volume of water required for the QuEChERS salt extraction tube was determined as 2.6 g using the following calculation:

74% of 10 g = 7.4 g

10 g – 7.4 g = 2.6 g

5.1.3 QuEChERS extraction blend

Other aspects of the QuEChERS protocol that were investigated included the kit composition. The Agilent salt extraction kits consisted of 50 mL centrifuge tubes with ceramic homogenisers, with salt sachets consisting of 4g of $MgSO_4$ and 1 g of NaCl. These were

selected given the density of the matrix and the option to better protect any thermally labile analytes of interest; the ceramic homogenisers are known to help break apart the matrix during the shaking/vortexing, and the salt sachets were preferable to pre-filled tubes as adding the salts directly to the matrix causes heat and can result in thermal decomposition.

An advantage in using the QuEChERS protocol is the flexibility of the dSPE clean-up stage. There are a several dSPE sorbents that can be used with the protocol, each targeting the removal of a specific interferences. With the high degree of lipophilicity anticipated with the matrix, dSPE kits containing C18 would seem to be highly suitable as this would remove interfering alkylated lipids, however, given the high hydrophobicity of HBCDD, three different dSPE kits were tested to find which, if any, gave optimal recoveries for HBCDD. Therefore, using the adjustments described above, three kits were tested (see Table 5.1) with the amended protocol described in Chapter 2, Section 2.5.3.

Table 5.1: QuEChERS kit composition and how it may be applied.

Kit	**Contents**	**Recommended application**
"General fruits and vegetables"	15 mL tubes + ceramic homogenisers with 400 mg of Primary and Secondary Amine (PSA) material and 1200 mg $MgSO_4$.	Removes polar organic acids and some sugars and some lipids
"Fruits and vegetables with fats and waxes"	15 mL tubes + ceramic homogenisers with 400 mg PSA, 400 mg C18 End Capped (EC) and 1200 $MgSO_4$	Removes polar organic acids, some sugars plus a wider range of lipids and sterols
"Drug residues in meat"	15 mL tubes + ceramic homogenisers with 150 mg C18 and 900 mg $MgSO_4$.	Removes biological matrix interferences, including hydrophobic substances such as lipids, plus proteins.

The results of the analysis showed that the HBCDD isomers were extracted and identifiable in the chromatogram. Interestingly, the "General fruits and vegetables" and "Fruits and vegetables with fats and waxes" QuEChERS kits (containing PSA sorbent) showed the best precision in extraction performance across the triplicate tests, while the "Drug residues in meat" QuEChERS kit gave inconsistent recoveries (see Appendix M). For this reason, the meat extraction kit was discarded for further work. Given the similarity of performance of the PSA-containing kits, and the need to future-proof the method for a potentially more lipid dense biota species, the "Fruit and vegetable with fats and waxes" kit was selected for further investigation. Disappointingly though, despite encouraging precision of performance for HBCDD recovery, there were markedly different recoveries for each HBCDD isomer; α and γ – HBCDD had far lower recoveries than β – HBCDD. As β – HBCDD eluted after α – HBCDD and before γ – HBCDD, we can assume that the hydrophobicity of β – HBCDD is between that of α and γ – HBCDD. Due to this, it is unlikely that the difference in recoveries between the three isomers was related to their respective affinities for the biota matrix and was more likely to be related to matrix interferences, either by suppression of α and γ – HBCDD during ionisation where compounds that co-eluted with these isomers were competing for ionisation, or enhancement of β – HBCDD from a co-eluting compound with a similar *m/z*.

To test this hypothesis, the volume of water added to the salt extraction step was increased to 5 mL with the same addition of 10 mL of ACN and another test with the addition of 10 mL of water and 15 mL of ACN was conducted (see Appendix N). Surprisingly, the modified addition of 5 mL of water resulted in a decrease in the recovery of β – HBCDD, rather than the expected increase in α and γ – HBCDD, which did not exhibit a change in response. This was further displayed with the 10 mL addition of water, with β – HBCDD response dropping again with the effect of additional ACN not apparent. These results suggest that the difference in HBCDD recovery may have been due to ion enhancement of β – HBCDD with the initial set of conditions, but the additional water may have encouraged further release of polar interferences from the biota which may have then caused ion suppression in the mass spectrometer. Further tests were conducted by analysing the extract after salt extraction only, to see what the difference in response was for the isomers with a 5 mL vs a 10 mL addition of water. The difference in recovery of β – HBCDD was striking, with recovery dropping by over 50% with the 10 mL addition compared to the 5 mL (see Appendix O), which again indicates that matrix effects are at play. QuEChERS is a broad process that would be more prone to

extracting interferences rather than a more refined procedure, but the simplicity and the significantly shorter preparation times are the compensations for this.

Subsequent tests were performed, again with 5 mL of water and 10 mL of water /15 mL of ACN added to the salt extraction step, only this time the volume of the ACN aliquot taken for clean-up was modified, with 2 mL taken from the tests with 5 mL of water added, and 4 mL taken from the tests with 10 mL of water. The logic behind this was that a lower volume of extract would mean a lower amount of matrix interferences, and potentially result in more consistent recoveries of the isomers. Interestingly, the results for the addition of 10 mL water and 15 mL ACN with 4 mL extract showed an increase in the β – HBCDD response, while the 5 mL addition of water with 2 mL of extract exhibited a poor recovery for all the isomers (see Appendix P). These results indicate that a 2 mL extract volume may be too low a ratio with the clean-up material which was apparent during preparation as the thin solvent layer could not be pipetted out of the centrifuge tube without taking up particles of the clean-up material. For a visual illustration of the alternative steps of this process see Appendix Q.

5.2 Concluding remarks

Homogenisation using the Omni kit produced a noticeably better homogenate with a smooth paste being created as opposed to the hand blender which by comparison still looked chunky and still contained some unblended pieces of skin. The milling process does produce quite a bit of heat however, so the method will need to be investigated further firstly to assess whether the heat has a significant effect on HBCDD, and further method development will be conducted to minimise the exposure of the biota to the heat, possibly via a cool water bath between each round of milling or potentially research into cryo cooling techniques.

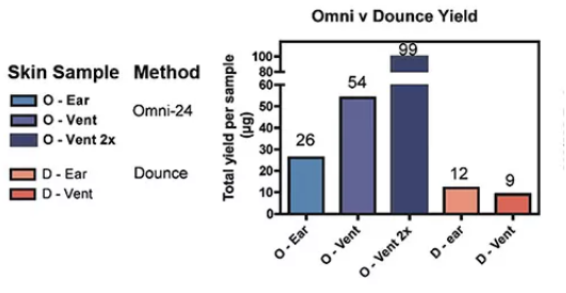
The QuEChERS tests show that HBCDD is extractable via this method, which is very promising as this was an uncertainty, considering the lipophilic nature of HBCDD and the QuEChERS method not being designed for fish matrix, the initial tests look promising.

5.3 Further work

Future testing will commence with the smaller dSPE QuEChERS tubes to try and determine the cause of the enhancement of the β – HBCDD peak; the dSPE kit for “Fruits and vegetables

with fats and waxes" is available as a 2 mL tube containing 50 mg of PSA, 50 mg of C18EC and 150 mg of $MgSO_4$. This may provide a more efficient extraction given the ratio of clean-up material to extract will be more suitable, as the lower volumes of extract needed to use the smaller tubes could reduce the intensity of matrix effects and hopefully allow the clean-up material to perform more efficiently[71]. If this test is unsuccessful then the investigation of alternative clean up procedures will commence, one potential example being addition of sodium acetate and chloroform, then sulphuric acid in subsequent clean up steps.[18]

The homogenisation efficiency of our current procedure will also be tested against the Omni Bead Ruptor Elite homogenisation on a more analytical level, with x11 replicate samples from each homogenisation source being spiked and extracted, testing the reproducibility of results across the homogenised matrix for each technique. On the Omni website they advertise that compound recoveries are greatly increased when using this technique (see Figure 5.1 below) and so it can be expected that the recovery of HBCDD should be far greater from the biota homogenised using the Omni kit than the hand blender. The results of these tests will potentially influence how biota will be prepared in our laboratory in the future.



Total mRNA yields and relative purity; Absorbance values at 260 nm and 280 nm were used to determine total mNRA yield and puity following extraction. mRNA yields where on average 4 times higher when bead milling was performed compared to dounce homogenization. Nucleic acid relative purity was comparable between both methods.

Figure 5.1: Comparison of mRNA yield obtained from dounce homogenisation and bead milling. Taken from the Omni International website https://uk.omni-inc.com/bead-ruptor-elite.html

6. CONCLUSION

HBCDD is a polybrominated flame retardant that has had a wide variety of applications, including upholstered furniture, packaging materials, domestic appliances, and extruded and expanded polystyrene in the building industry. As a compound that is now considered a priority substance under the Environmental Quality Standard (EQS) Directive, the purpose of this project was to develop and validate methods to analyse and quantify the concentration of HBCDD in river waters and saline waters as well as in biota (fish) matrix to allow the routine monitoring of Welsh environmental waters. This was achieved for the aqueous matrices, using Affinisep hydrophilic – lipophilic balance (HLB) solid phase extraction (SPE) disks to load the sample, elution of the analyte into methanol and a novel pre-concentration step using 'Flip-Flop' tubes and a Genevac Rocket evaporator. The analysis of HBCDD by liquid chromatography mass spectrometry (LC-MS) using a triple quadrupole system was also successful.

Comparison tests between the ESI source and the APCI source showed that the sensitivity of the ESI source was significantly higher. A comparison exercise between several reverse-phase columns with various manufacturers resulted in the Agilent EC-C18 column being selected, it provided good chromatography and acceptable resolution between the peaks. The Agilent LC-MS system itself was found to be robust, providing reliable, repeatable results when tested and considering the analysis of HBCDD is to be performed on a routine basis in the laboratory, these factors are essential and the decision to use the Agilent system for validation was the logical choice.

Method development within the Natural Resources Wales Analytical Services (NRWAS) laboratory must be followed by an extensive validation process that has been designed so that it satisfies UK regulatory criteria. The saline and freshwater methods both successfully passed validation with the data showing a good level of accuracy and precision even when the surrogate standards had lower levels of recovery than in other published studies, which emphasises the advantage of having a labelled surrogate standard for each of the HBCDD isomers as the loss of surrogate standard would be so similar to that of the analyte that the calculated final concentrations, which take into account any surrogate standard loss, were highly accurate. The freshwater method was tested using real routine river water samples where concentrations were found up to ~0.49 ng/L, exceeding the LOQ, giving justification

that HBCDD monitoring in the environment is currently required and confirming that the method developed is fit for purpose. Development of the biota method will now be a priority, as due to the lipophilic nature of HBCDD it can be predicted that the bioaccumulation will be more evident in this matrix.

Development of the biota method has begun with promising results with refining the blending technique of the matric to create a truly homogenous sample source. The initial visual findings are very promising as the Omni homogeniser produced a very fine paste, in stark contrast to the hand blended matrix which was very coarse and still contained bits of skin and bone after blending, though further tests will need to be done to ensure that the heat produced during blending does not adversely affect the sample. Comparison tests between the Omni homogeniser versus the hand blender will produce interesting data and should the Omni provide the better results the time saved by using this piece of equipment will be substantial, significantly shortening the sample preparation time, for the HBCDD method and other methods involving biota matrix going forward.

The initial testing of HBCDD extraction using the QuEChERS method is very promising, the three main HBCDD isomers appeared in the chromatogram of the analysed extract which tells us that despite the lipophilic, non-polar nature of HBCDD, it can be extracted using this method. The next steps will be to investigate how to remove the matrix interferences that do not appear to be removed efficiently by the standard clean-up procedure of the QuEChERS method, so alternative methods must be explored, with the intention of keeping the method as simple as possible.

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8. APPENDICES

Appendix A: Disassembled 'Flip-Flop' tube

Figure A.1

Appendix B: Calculations and statistics used for method validation

Figure B.1: The following statistical parameters are used to assess method performance during validation:

M – Matrices

L – Level

R – Replicate

N or B – batch

$\sum \overline{X}^2$ MDT Sum Mean Square - Avg replicate values for a level - squares

Sums everything for batch = Sum of square of means

MDT Sbw^2 $\left(\sum \overline{X}\right)^2$

$$\sqrt{\frac{\left(\sum \overline{X}^2\right)-\left(\sum \overline{X}\right)^2}{N-1}} \qquad (\text{N} = \text{no batches})$$

Within batch SD

$$\text{Total Variance} = \frac{\left(\sum X - \overline{X}\right)^2}{\text{N - 1}}$$

$$\sqrt{\left(\frac{\text{Cumulative total of sum of variance}}{\text{Total batches}}\right)} - \text{Sw}(\text{Within batch SD})$$

Between batch SD (Sb)

$$\sqrt{\left(\left(\text{Sbw}^2\right)^2 - \left(\frac{(Sw)^2}{2}\right)\right)}$$

Total SD

$$\sqrt{\left(Sw^2 + Sb^2\right)}$$

MDT LOD $\qquad (\text{t} = \text{A } 10\% \text{ significan ce standard value at N - 1 data sets (DoF)})$

$$\left(\left(2\sqrt{2}\right)t\right)Sw$$

RSD

$$\left(\frac{\text{Total SD}}{X}\right)100$$

DoF

- Rounds up above 0.5

$$\frac{(n(n-1))\left(2\left(Sbw^2\right)^2+\left((n-1)Sw^2\right)^2\right)}{4n\left(Sbw^2\right)^4+\left((n-1)R(Sw)^4\right)}$$

% Bias

$$\left(\left(\frac{\text{Average}}{\text{Nominal}}\right)-1\right)100$$

Recovery

equation 2 -

$$\text{Blank spiked equiv} = \left(\frac{\text{conc of spiking solution into blank}}{\text{conc of spiking solution at spike level}}\right)(\text{Amount of spiking solution in blank})\ \text{X}\ \left(\frac{\text{Amount of matrix material in spike}}{\text{Amount of material in blank}}\right)$$

Blank spiked equiv taken off blank amount and spiked amount used in equation and so the blank is zero

Recovery – $Eq2$

$$100\left(\text{Mean x total amount material (including spike) -}\left(\frac{\text{Average blank x Amount matrix}}{\text{Spiking conc x Spiking amount}}\right)\right)$$

Eq4

Blank spike equiv as eq2

E x F factors used in overcall equation

$$E = \frac{\left(\left(\text{Average x total amount}^2\right) - \text{Average blank x Amount matrix}^2\right)}{\text{Spiking conc x Spiking amount x Total amount}}$$

$$F = \frac{\left(\text{Average blank x amount matrix}\left(\text{total amount - matrix amount}\right)\right)}{\text{Spiking conc x Spiking amount x Total amount}}$$

Recovery – In Dave Wilsons Documents

$$SD\left(E + \sqrt{\left(E^2 - 4F\right)}\right)$$

$$\text{UoM of Variance} \rightarrow \text{Variane x Dof} = \text{Varriance component of UoM}$$

RSD^2 x DoF

DoF of Bias of UoM - Uses Bias from recovery if poss but overall Bias x DOF % if not

LoD bounds down to 1sf only if the resulting LoD is < 10% lower than the original.

F Statistics Value

$$\frac{2 \text{ x } Sbw2^2}{Sw^2}$$

Significan ce of between batch SD

x uses value of F
100 x (1 - F) = Level of significan ce

Calculates F at 90% significan ce assumming infinite DoF

LoD - 3 Rules

Rule 1 - If $\frac{\text{LoD}^2}{\text{Target}^2} < F \text{ (at 90\% sig)} \rightarrow \text{passes}$

Rule 2 - If $\text{LoD} = 0 \rightarrow \text{passes}$

Rule 3 - If $\text{Target} = 0 \rightarrow \text{passes}$

RSD Evaluation - 4 rules

Rule 2 only in use if rule 1 is false

Rule 1 - $\frac{\text{RSD}^2}{\text{RSD(Target)}^2} < \text{F at 90\% significan ce}$

Rule 2 - If target is not $0 = \frac{\text{Total SD}^2}{\left(\frac{(\text{MRV Target})^2}{16}\right)} < \text{F} = \text{ok}$

Rule 3 - If $\text{RSD} < \text{target} \rightarrow \text{passes}$

Rule 4 - If nominal value $= 0 \rightarrow$ passes (No RSD failures on blank)

Evaluation of % Bias - 4 rules

Rule 1 - If % value is $< \text{target} + \left(\frac{\text{RSD x t (at 90\% sig)}}{\sqrt{\text{n}}}\right) = \text{passes}$

Rule 2 - If nominal - mean < 5 MRV target = go to Rule 3

Rule 3 - Nominal - mean $< \frac{\text{MRV target}}{2} + \frac{\text{Total SD x t}}{\sqrt{\text{n}}} = \text{passes}$

Rule 4 - If $\text{target} = 0 \rightarrow \text{passes}$

Evaluation of Absolute Bias - 2 rules

Rule 1 - Average - Nominal $< \frac{\text{MRV target}}{2} + \text{Total SD x t (at 90\% for no batches)}$

Rule 2 - If $\text{target} = 0 \rightarrow \text{passes}$

Standard Deviation of means Batch Recovery

- Additional Recovery evaluation data

SDM Batch rec returns 2 values $\rightarrow$ V1 + V2
Calculates value
V1 = Average mean recovery

$$V2 = \frac{\sqrt{\text{Total variance of recoveries}}}{n}$$

$$\text{then } 100 \text{ x } \frac{V2}{V1} = \text{mean recovery RSD}$$

$$\frac{\text{Mean recovery RSD}}{\sqrt{\text{no of batches}}} = \text{mean recovery standard error}$$

mean rec standard error x value of t at 90% for no of batches
= mean rec 90% confidence internal.

recovery range - recocery $\pm$ confidence interval

Evaluation of Recovery - 2 rules

$$1 - \text{If absolute of LOD - recovery is} < \text{recovery target} + \left(\frac{\left(100 \text{ x } \frac{V2}{V1} \right)}{\left(\sqrt{n} \right) t \text{ at } 90\%} \right) \rightarrow \text{passes}$$

2 - If target = 0 $\rightarrow$ passes

UoM
If more than 1 level and is greater than 1 (i.e. not the blank) then UoM DoF is the standard DoF.
But if it is level 1 or there is 1 level also uses standard DoF

UoM (RSD) =

$$\sqrt{\frac{\text{Total DoF (Variance component)}}{\text{Total DoF overall}}} \rightarrow \text{UoM} = \text{Variance x DoF} \rightarrow \text{not the same as overall DoF}$$

UoM varriance x D0F - uses DoF relevant to each level

UoM (RSD) =
UoM Bias = (DoF) x Bias

At maximum level calculates overall VoM

$$= \frac{\text{Total of Uom (Bias x DoF)}}{\text{DoF (total)}}$$

For 1 level not possible to calculate UoM, Bias, uses % Bias

$$\text{UoM RSD} = \frac{\text{Total UoM (Var x DoF)}}{\text{DoF (total)}}$$

Overall UoM for Matrix = (UoM(RSD) x 2) + VoM (Bias)

$$\text{Total of RSD UoM} = \sqrt{\frac{\text{(Total UoM(var x DoF)}}{\text{Total DoF}}}$$

$$\text{Total Bias UoMs} = \sqrt{\frac{\text{(Total UoM(Bias x DoF)}}{\text{Total DoF}}}$$

UoM total = 2 x RSD + Bias

The final concentration data is used to assess the validation; this can be either entered manually or imported in from an Excel-compatible data files.

Appendix C: Responses for different flow rates tested during instrument optimisation on the Shimadzu system.

Table C.1

Flow rates (L/min)			**Average peak height from 3 replicates**	**Standard Deviation of 3 replicates**
Nebuliser gas	Heating gas	Drying gas		
1.0	3.0	7.0	704,587	26,399
1.5	3.0	7.0	868,167	27,160
2.0	3.0	7.0	1,026,521	25,184
1.0	3.0	9.0	688,146	11,097
1.5	3.0	9.0	883,834	43,951
2.0	3.0	9.0	1,048,496	4,668
1.0	3.0	11.0	711,964	4,528
1.5	3.0	11.0	890,391	19,564
3.0	5.0	11.0	872,996	5,823
2.0	3.0	13.0	834,382	10,507
2.5	3.0	13.0	870,069	8,592
3.0	3.0	13.0	848,926	17,958
2.0	4.0	13.0	828,112	14,987
2.5	4.0	13.0	871,123	3,463
3.0	4.0	13.0	876,198	5,588
2.0	5.0	13.0	837,306	6,931
2.5	5.0	13.0	872,099	7,835
3.0	5.0	13.0	863,301	330
2.0	3.0	15.0	794,324	7,358
2.5	3.0	15.0	831,286	14,165
3.0	3.0	15.0	828,468	4,830
2.0	4.0	15.0	819,370	5,548
2.5	4.0	15.0	853,116	6,289
3.0	4.0	15.0	852,410	10,728
2.0	5.0	15.0	808,061	5,448
2.5	5.0	15.0	849,986	11,666
3.0	5.0	15.0	852,403	11,897

Appendix D: Plot of resolution for different mobile phase flow rates in APCI

Figure D.1: Resolution between α and β isomers

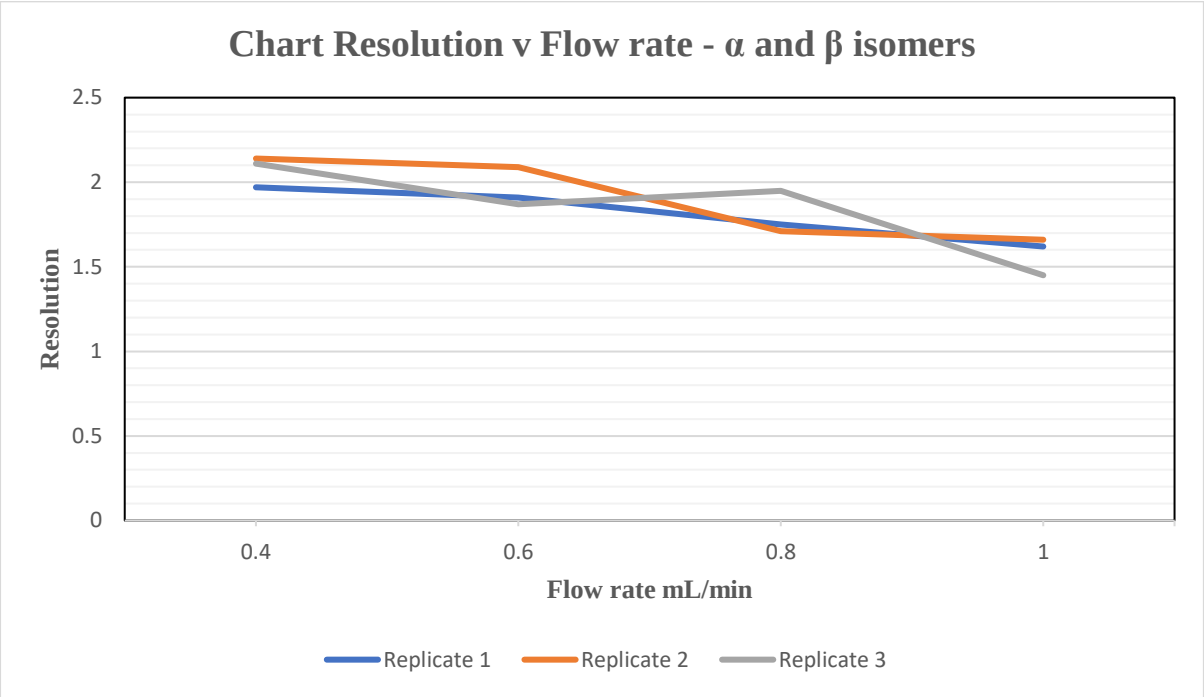
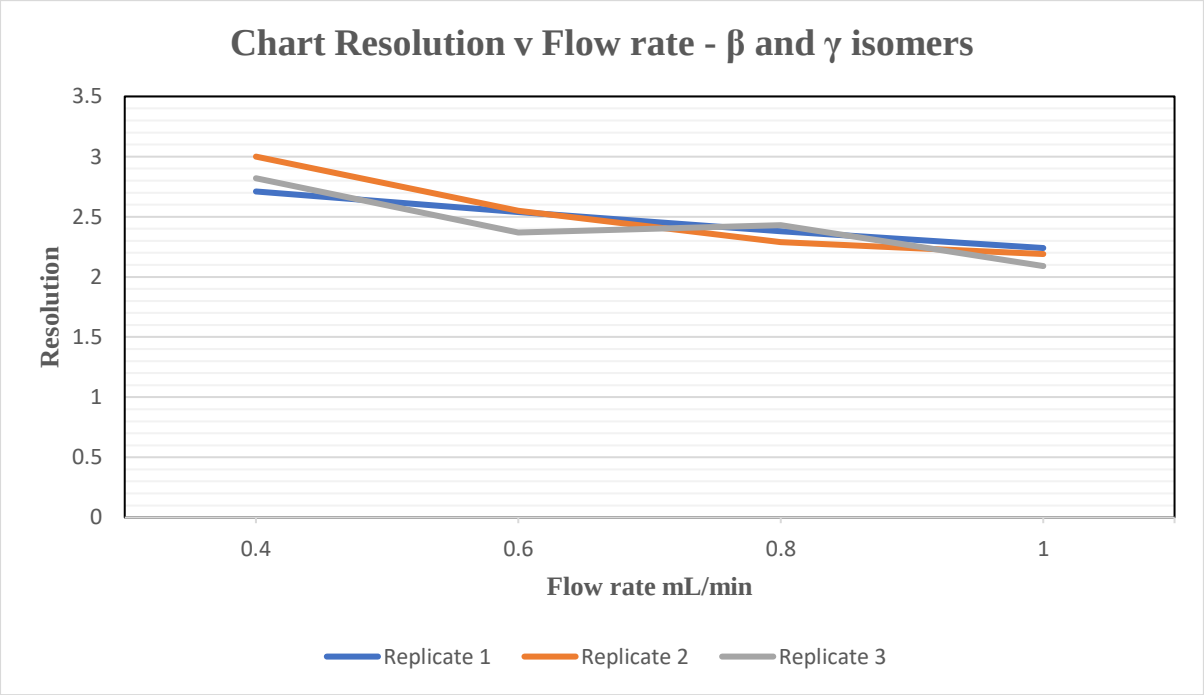


Figure D.2: Resolution between β and γ isomers



Appendix E: Chromatograms showing retention times and peak shapes of HBCDD isomers using 50:50 methanol and acetonitrile as the organic mobile phase

Figure E.1: α, β and γ -HBCDD analysed as separate solutions

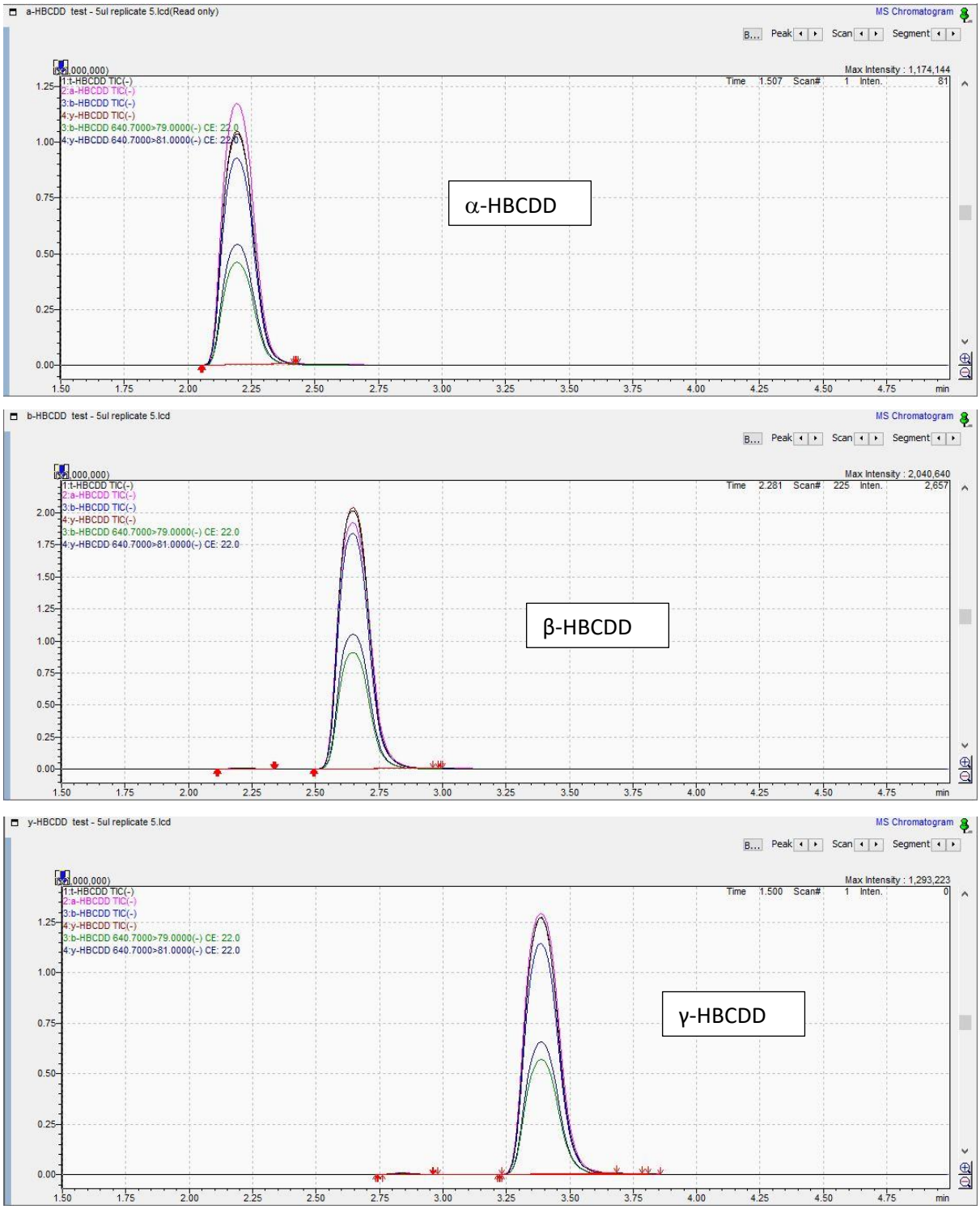
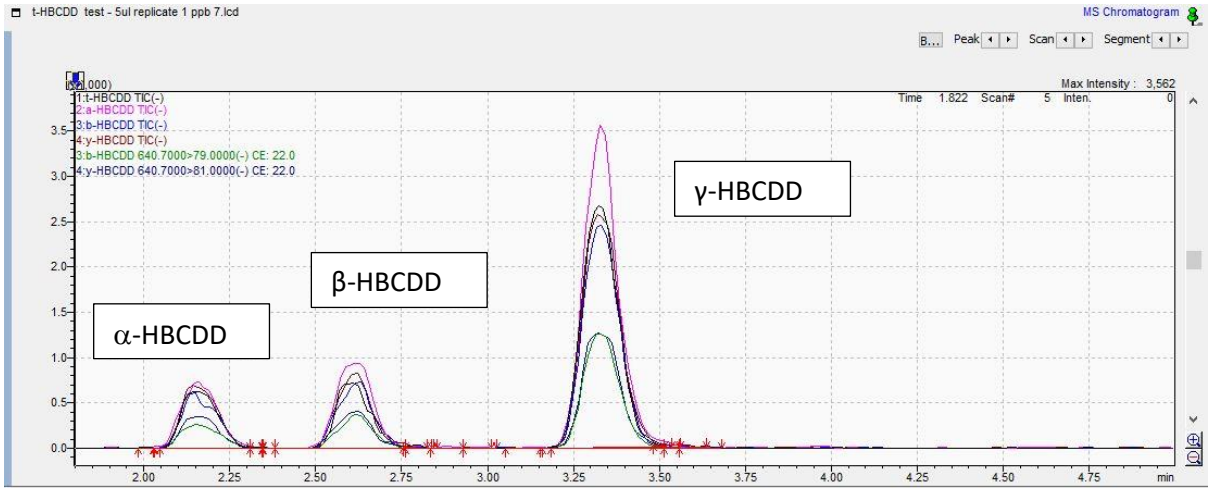


Figure E.2: α, β and γ -HBCDD analysed as a mixed solution



Appendix F: Results of Agilent instrument evaluation replicate injections with % RSD and IDL calculations

Table F.1

Injection Number	Peak Area
1	34.45
2	28.04
3	24.17
4	34.40
5	36.51
6	39.10
7	37.80
8	31.91
9	34.38
10	28.28
11	36.06
Mean	33.1909
Standard Deviation (SD)	4.6210
% RSD	13.92
IDL	**3.1**

Appendix G: Agilent repeatability test with reinjections every hour over 12 hours, conducted during instrument evaluation

Table G.1

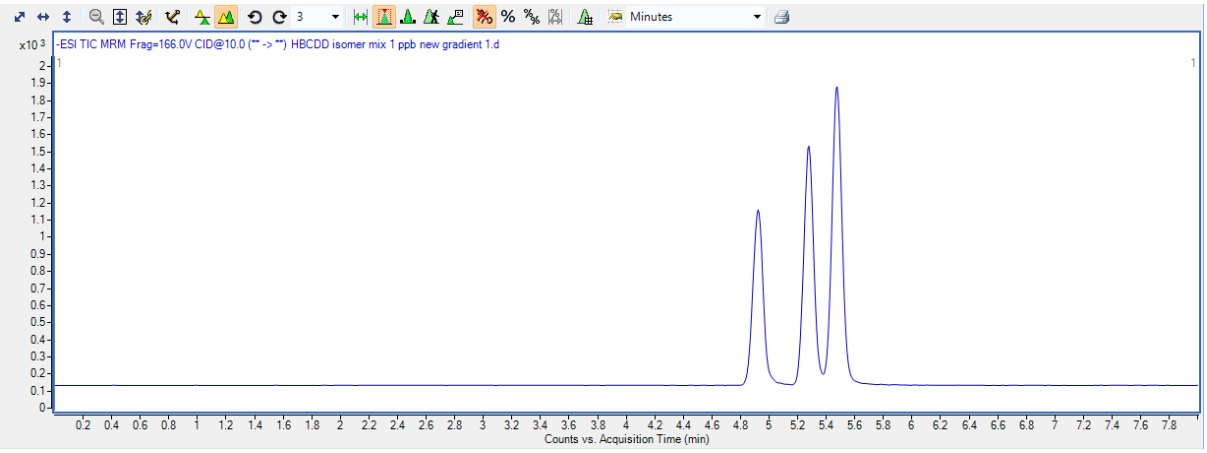
Time passed (hours)	**Peak Area**
0	126.61
1	100.28
2	116.26
3	101.08
4	117.00
5	122.66
6	142.36
7	128.33
8	113.03
9	129.64
10	126.83
11	120.49
12	118.59
Mean	120.2431
SD	11.4731
%RSD	**9.54**

Appendix H: Gradient and chromatography showing 1st set of changes from original evaluation gradient

Table H.1

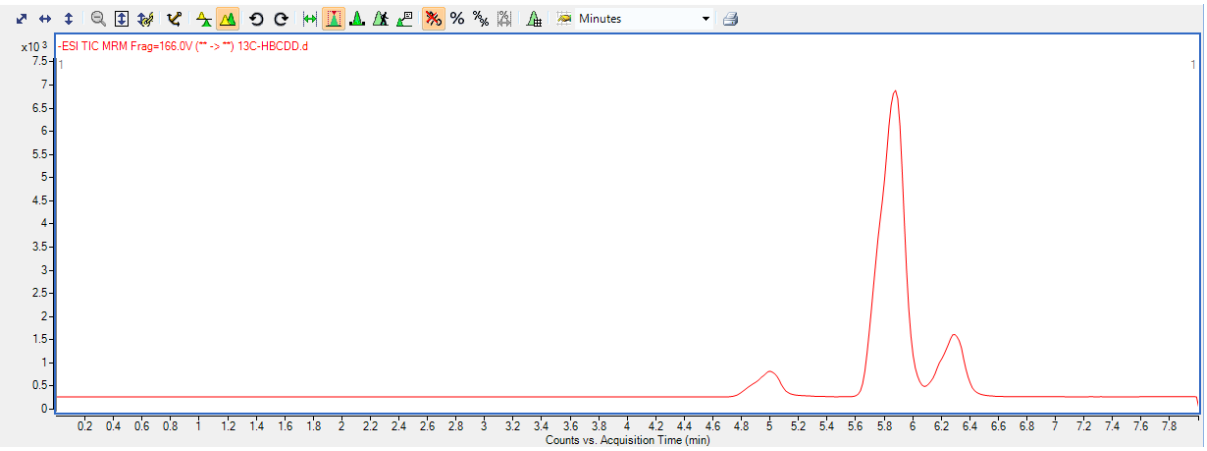
Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	20.0	80.0	0.350	500.00
4.00	10.0	90.0	---	---
7.00	5.0	95.0	---	---
8.00	5.0	95.0	---	---
9.00	20.0	80.0	---	---

Figure H.1



Appendix I: Chromatography of all 3 isomers based on transferred Shimazu gradient method.

Figure I.1



Appendix J: Excerpt from the NRWAS Quality Control procedure, including acceptance criteria for validation of a method

Figure J.1

PERFORMANCE TESTING

Introduction

It is necessary to establish the performance characteristics of an analytical system (e.g. LoD, Precision, Accuracy and UoM) as a means of demonstrating adequacy and as the basis for continuing within laboratory routine quality control. The accepted approach for water laboratories is described in NS30. The basic principles of this approach can be extended, and with modification, be applied to other types of matrix, such as waste, contaminated land and other solid samples. For many solid matrices, suitable certified reference materials (CRM's) and derived local reference materials (LRM's) are available and can be used as part of the performance testing programme design.

Note CRM's are also referred to as Standard Reference Materials (SRM's)

Newly established analytical systems/methods must be performance tested before being accepted for routine use. The Quality Specialist on a regular basis will review performance data (including routine AQC data), and whenever significant system change has occurred.

If applicable, where other standards, e.g. MCertS, specifies a performance testing protocol and targets then these will be followed.

3.2.2 Application and Organisation of Performance Testing

NRW Analytical Services customers require that 'The analytical system must have been tested to demonstrate that performance characteristics specified have been achieved'.

All methods in routine use at a laboratory will have performance data, which demonstrates that specified performance characteristics required for a parameter is achieved.

Performance testing design should be based on the approach of NS30.

Performance testing will establish:

- the LoD obtained by the analytical system;

- the precision obtained over the applicable range;

- the accuracy (recovery or bias) from specific matrices;

- the Minimum Reporting Value (MRV) is set as the LoD rounded up to 1 significant figure unless the 1/10th critical value of interest (e.g. Consent, regulatory value or other agreed target) is not being met, then the LoD may be rounded up to 2 significant figures (e.g. COD 12 mg/l).

Performance Testing shall be carried out under the direction of the appropriate Team Manager, Technical Specialist or Quality Manager. The Quality Manager will ensure that the exercise is carried out in accordance with the requirements of quality documentation. Where possible the exercise should be run with routine samples in normal batches.

3.2.3 Data Processing

All 'raw' data relating to performance testing should be retained. The raw data should be processed by a suitable software package capable of analysis of variants (ANOVA) as outlined in NS30.04

A basic suitable package is marketed by WRc plc or an in-house equivalent spreadsheet may be used particularly for multiple parameter suites (e.g. Organic suites)

Processed performance testing results are retained for a minimum of 6 years.

3.2.4 Acceptability Criteria

Guidance on acceptability criteria is included in the Work Instruction in NRW Analytical Services Quality Control Procedures 3.1 and 3.2. - Performance Testing Waters and Performance Testing Solids.

Inspection and verification of data, including the treatment of outliers, and the assessment of acceptability, will be carried out by the Quality Manager, who will report and discuss findings to the appropriate Team Manager or Technical Specialist.

Validation data for new methods and significant changes to existing methods must be formally assessed against customer and/or regulatory targets (when available) and signed off as fit for purpose by a chemistry Team Manager and by a member of the Quality team.

Appendix K: Surrogate standard % recoveries of validation samples

Table K.1: Saline validation batch for α-HBCDD

	ISTD % Recoveries - α-HBCDD $^{13}C_{12}$							
Validation batch number	SW blank 1	SW blank 2	SW LOQ 1	SW LOQ 2	SW Low spike 1	SW Low spike 2	SW High spike 1	SW High spike 2
1	38.1	28.0	38.7	29.2	35.4	29.4	31.4	33.0
2	43.4	41.4	30.5	44.6	42.5	34.2	41.3	42.8
3	38.2	31.4	46.7	41.0	38.1	39.5	39.7	35.8
4	48.4	50.1	52.2	43.8	20.3	54.2	51.3	45.3
5	40.9	36.0	46.2	43.7	43.0	44.1	57.6	51.2
6	52.9	44.2	41.7	59.8	45.9	39.9	55.7	43.5
7	40.9	41.6	38.7	12.1	38.1	33.8	38.9	49.1
8	38.1	43.3	40.2	44.6	48.8	12.0	48.3	45.5
9	42.6	42.2	42.7	47.6	48.1	47.0	37.9	35.4
10	45.9	41.0	49.0	51.7	49.8	52.0	45.7	49.2
11	50.8	50.8	34.4	47.1	43.3	34.0	48.2	45.3

Table K.2: Saline validation batch for β-HBCDD

	ISTD % Recoveries - β-HBCDD $^{13}C_{12}$							
Validation batch number	SW blank 1	SW blank 2	SW LOQ 1	SW LOQ 2	SW Low spike 1	SW Low spike 2	SW High spike 1	SW High spike 2
1	39.2	36.5	36.5	33.7	39.5	24.9	36.0	36.9
2	38.0	40.1	25.9	31.1	36.8	26.6	34.9	37.5
3	28.1	28.9	33.4	35.8	40.7	35.6	40.3	25.6
4	48.7	54.3	59.0	42.7	23.2	63.4	60.2	51.6
5	43.1	33.2	39.2	41.5	39.1	37.5	51.6	54.2
6	60.6	50.1	49.4	63.6	55.2	40.6	59.2	49.4
7	41.0	40.6	34.4	12.6	31.3	31.3	35.1	38.4
8	37.7	39.4	38.8	33.1	15.5	40.6	46.8	31.5
9	35.3	40.0	44.1	45.3	48.6	37.4	39.7	28.7
10	36.7	46.9	41.6	57.5	40.0	62.4	47.6	49.7
11	51.1	50.0	33.7	48.6	46.4	31.8	44.7	45.2

Table K.3: Saline validation batch for γ-HBCDD

	ISTD % Recoveries - γ-HBCDD $^{13}C_{12}$							
Validation batch number	SW blank 1	SW blank 2	SW LOQ 1	SW LOQ 2	SW Low spike 1	SW Low spike 2	SW High spike 1	SW High spike 2
1	38.4	37.2	36.6	37.2	37.8	25.1	35.8	34.4
2	42.7	42.8	23.7	35.0	44.1	34.7	41.3	37.8
3	36.7	32.6	41.4	40.9	37.3	35.2	41.2	31.0
4	48.8	60.7	61.4	59.6	25.3	57.9	55.3	55.7
5	48.4	34.0	41.9	36.2	38.6	35.0	55.1	58.7
6	60.2	52.4	47.0	60.9	60.5	42.3	56.4	47.9
7	40.7	39.7	44.0	11.3	43.9	34.8	36.4	42.9
8	41.6	44.2	44.9	36.7	15.9	46.9	45.7	37.7
9	35.6	41.1	42.6	43.4	47.9	37.3	36.2	25.5
10	42.7	46.2	43.7	53.9	44.9	55.7	41.3	47.1
11	41.6	42.5	28.8	41.3	44.4	31.8	38.5	42.4

Table K.4: Freshwater validation batch for α-HBCDD

	ISTD % Recoveries - α-HBCDD $^{13}C_{12}$							
Validation batch number	FW blank 1	FW blank 2	FW LOQ 1	FW LOQ 2	FW Low spike 1	FW Low spike 2	FW High spike 1	FW High spike 2
1	34.7	32.9	34.7	39.4	37.6	28.8	36.6	24.1
2	19.5	32.3	39.3	32.0	30.7	32.4	2.6	37.6
3	25.5	29.7	36.4	12.5	32.4	31.5	32.7	29.3
4	37.5	33.1	17.2	43.7	40.0	41.8	46.7	40.6
5	32.8	42.2	39.2	44.0	39.3	38.3	15.4	33.1
6	43.5	42.0	40.2	37.2	42.6	41.2	28.2	34.6
7	49.1	44.4	47.3	40.9	45.8	51.4	53.8	42.2
8	47.7	45.4	51.9	42.1	42.5	43.1	40.8	45.4
9	46.7	52.4	11.7	42.9	46.1	45.2	50.0	48.5
10	21.3	47.1	19.4	49.3	52.7	52.2	44.7	38.8
11	45.2	37.7	13.4	9.4	22.5	40.4	40.5	40.6

Table K.5: Freshwater validation batch for β-HBCDD

	ISTD % Recoveries - β-HBCDD $^{13}C_{12}$							
Validation batch number	FW blank 1	FW blank 2	FW LOQ 1	FW LOQ 2	FW Low spike 1	FW Low spike 2	FW High spike 1	FW High spike 2
1	38.7	36.8	39.0	40.4	38.5	31.7	38.8	22.4
2	19.0	34.7	38.1	42.7	36.4	36.0	3.0	38.1
3	26.8	26.7	35.1	13.3	33.0	29.3	34.5	32.7
4	43.9	43.1	19.6	49.9	42.6	52.4	50.4	47.0
5	33.4	45.8	39.7	49.2	42.7	36.6	17.1	37.1
6	43.8	43.6	45.1	36.2	44.3	43.1	28.0	38.4
7	55.4	50.5	47.1	46.9	49.8	57.4	57.8	41.2
8	54.5	46.4	54.7	44.0	46.9	44.9	43.6	46.1
9	47.6	58.2	12.9	42.2	47.5	45.8	47.3	55.8
10	20.0	46.8	20.1	57.0	55.2	61.3	47.4	41.4
11	44.6	41.4	20.0	9.2	20.5	42.9	41.7	43.4

Table K.6: Freshwater validation batch for γ-HBCDD

	ISTD % Recoveries - γ-HBCDD $^{13}C_{12}$							
Validation batch number	FW blank 1	FW blank 2	FW LOQ 1	FW LOQ 2	FW Low spike 1	FW Low spike 2	FW High spike 1	FW High spike 2
1	37.1	34.0	37.3	41.8	40.0	29.5	35.5	20.0
2	18.3	36.4	39.9	34.5	37.9	39.9	3.9	36.3
3	27.6	27.5	32.5	13.0	31.1	28.0	35.6	31.9
4	42.6	44.7	17.4	46.4	44.4	50.2	52.6	45.9
5	27.8	40.7	36.6	44.1	36.1	34.8	17.1	33.9
6	41.5	43.6	44.1	34.5	39.9	39.7	28.0	35.1
7	50.2	45.1	45.2	45.8	48.1	49.2	55.2	42.5
8	49.9	44.0	51.2	47.6	45.0	42.1	42.3	46.3
9	44.8	51.6	12.0	39.3	46.0	45.5	46.8	48.6
10	18.5	45.1	18.6	53.0	48.2	54.6	41.2	37.3
11	40.1	36.1	17.1	8.0	21.3	39.1	37.8	42.7

Appendix L: Chromatograms of different ratios of acetonitrile and methanol as mobile phase B

Figure L.1: 90% ACN and 10% MeOH as mobile phase B

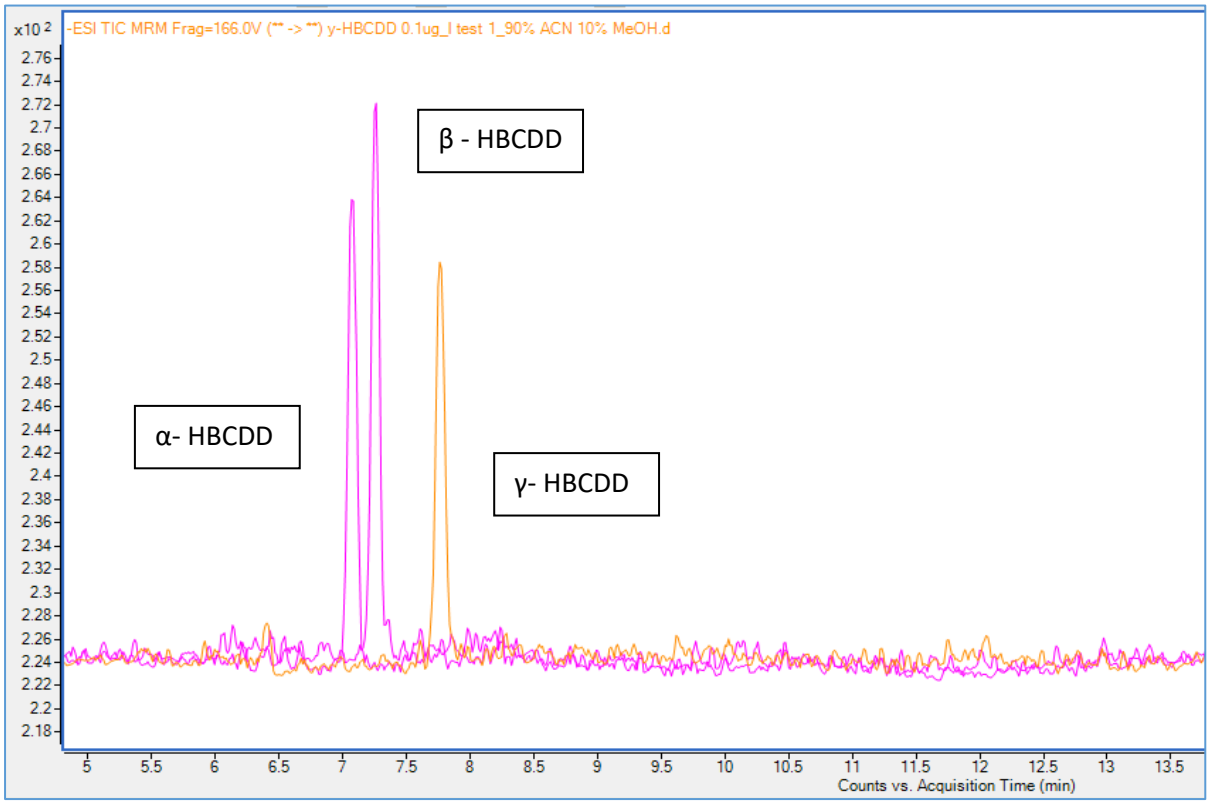


Figure L.2: 80% ACN and 20% MeOH as mobile phase B

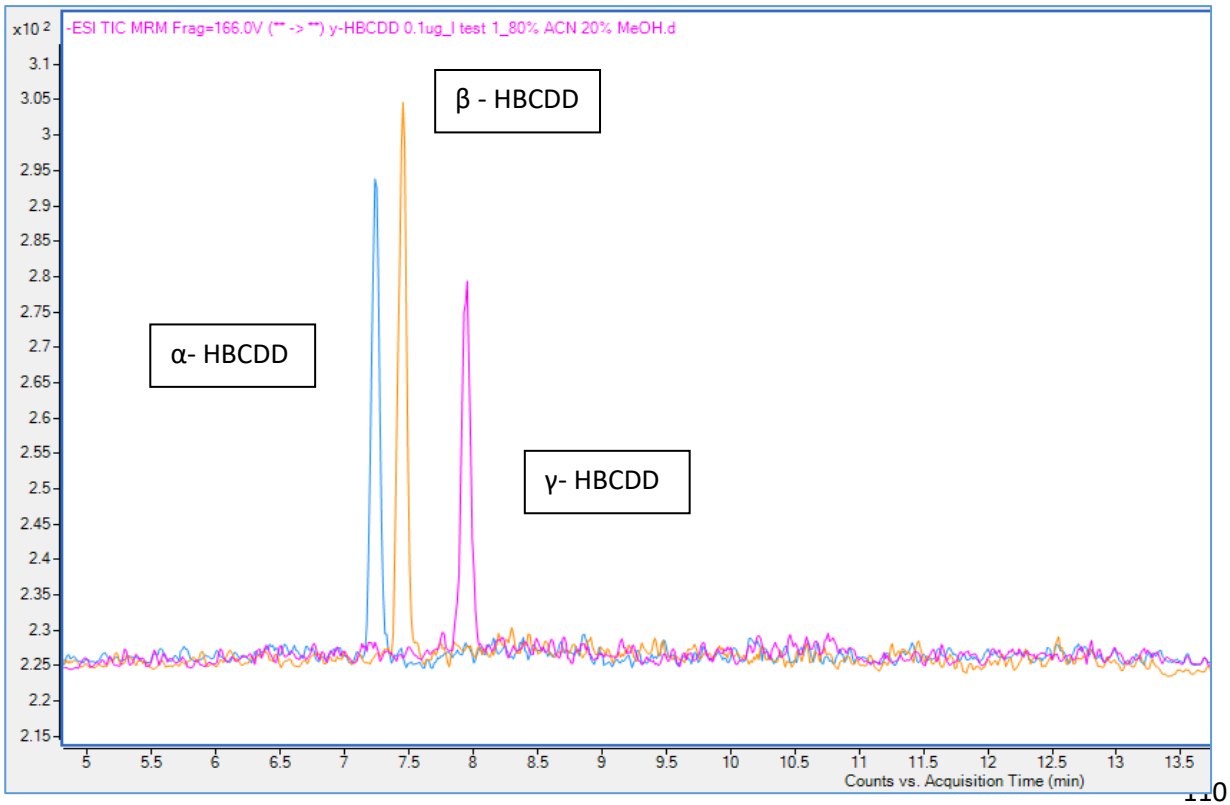


Figure L.3: 70% ACN and 30% MeOH as mobile phase B

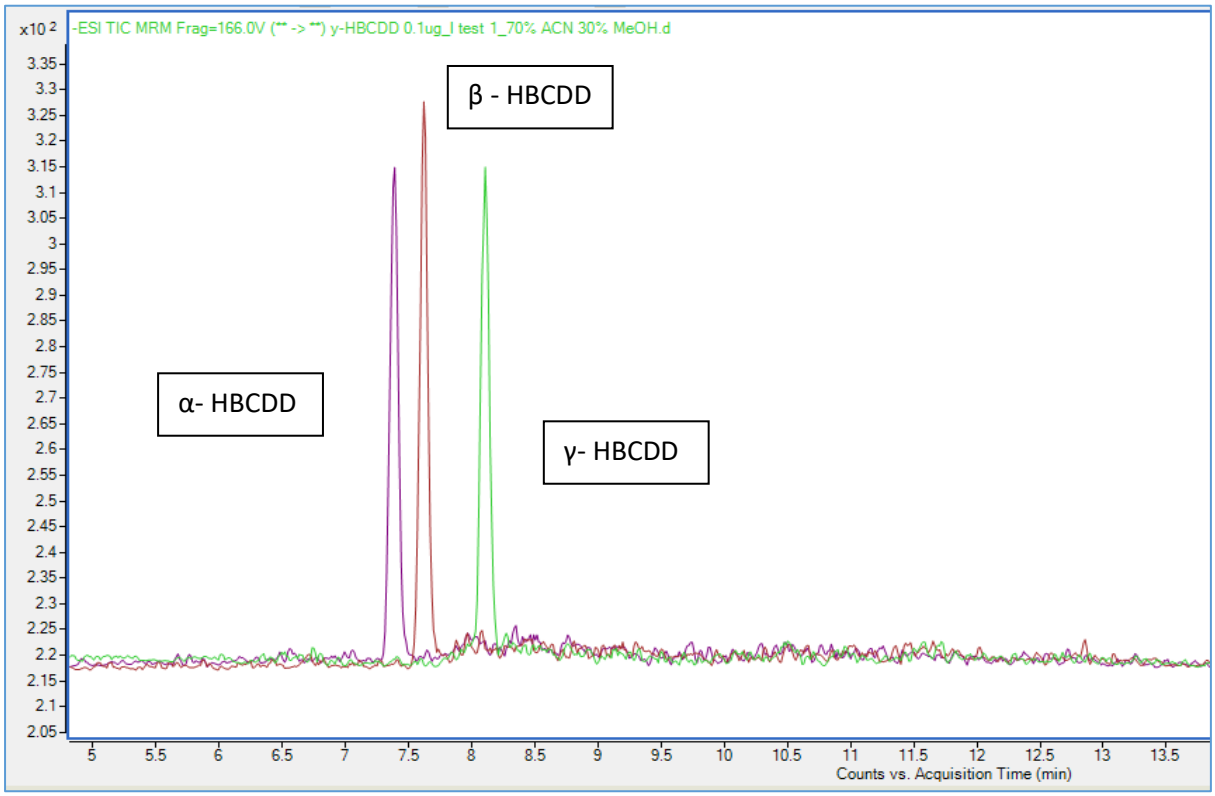


Figure L.4: 60% ACN and 40% MeOH as mobile phase B

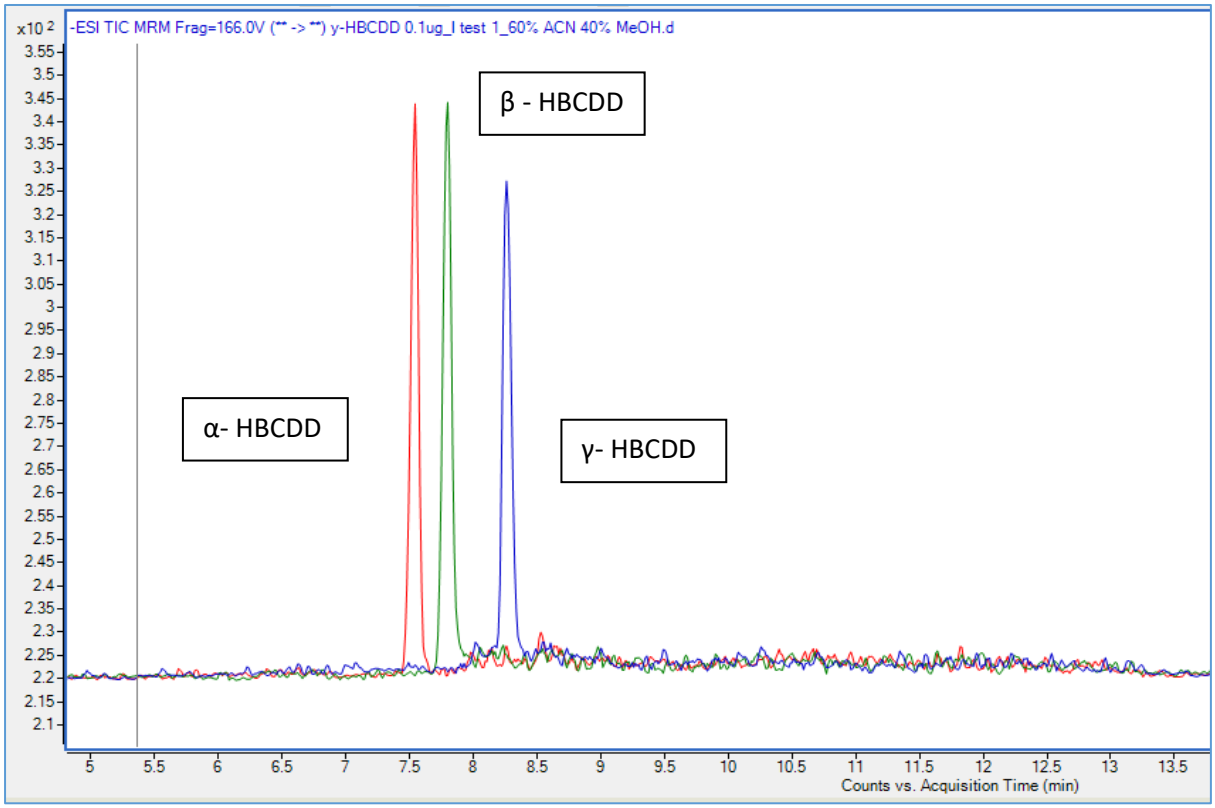


Figure L.5: 50% ACN and 50% MeOH as mobile phase B

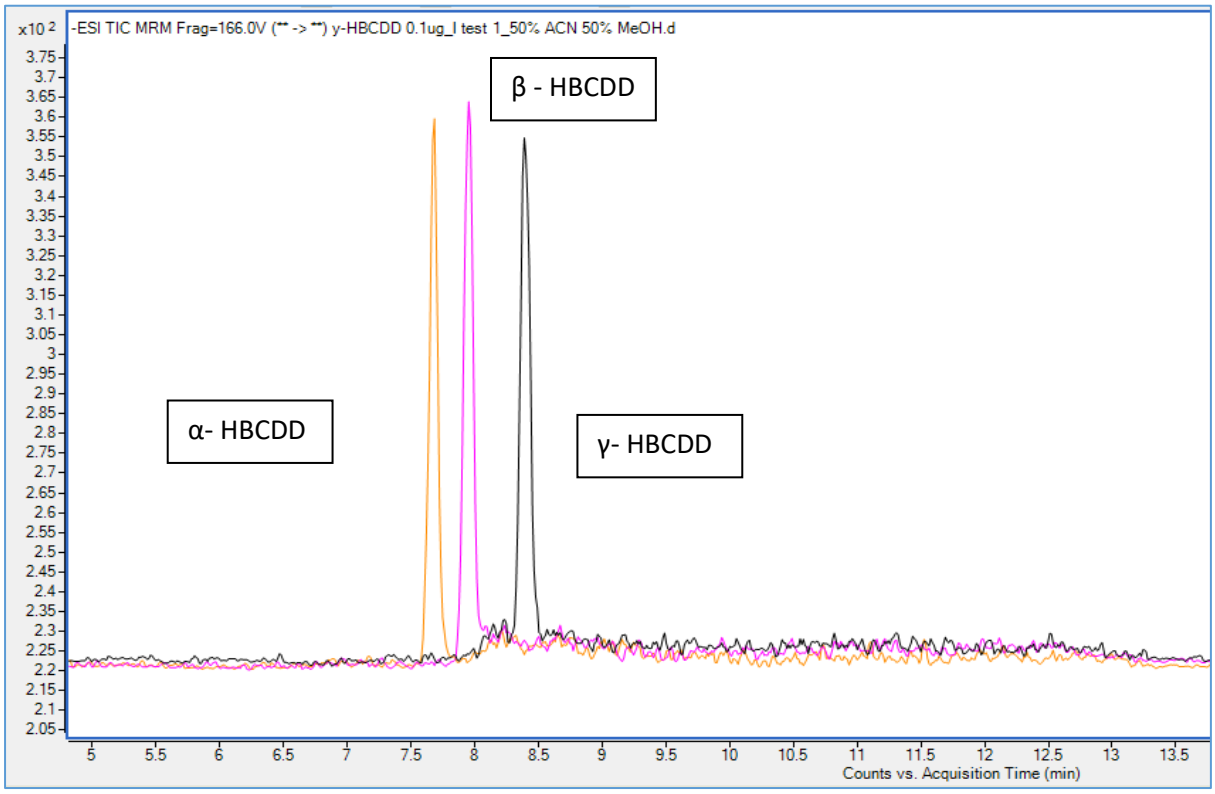


Figure L.6: 40% ACN and 60% MeOH as mobile phase B

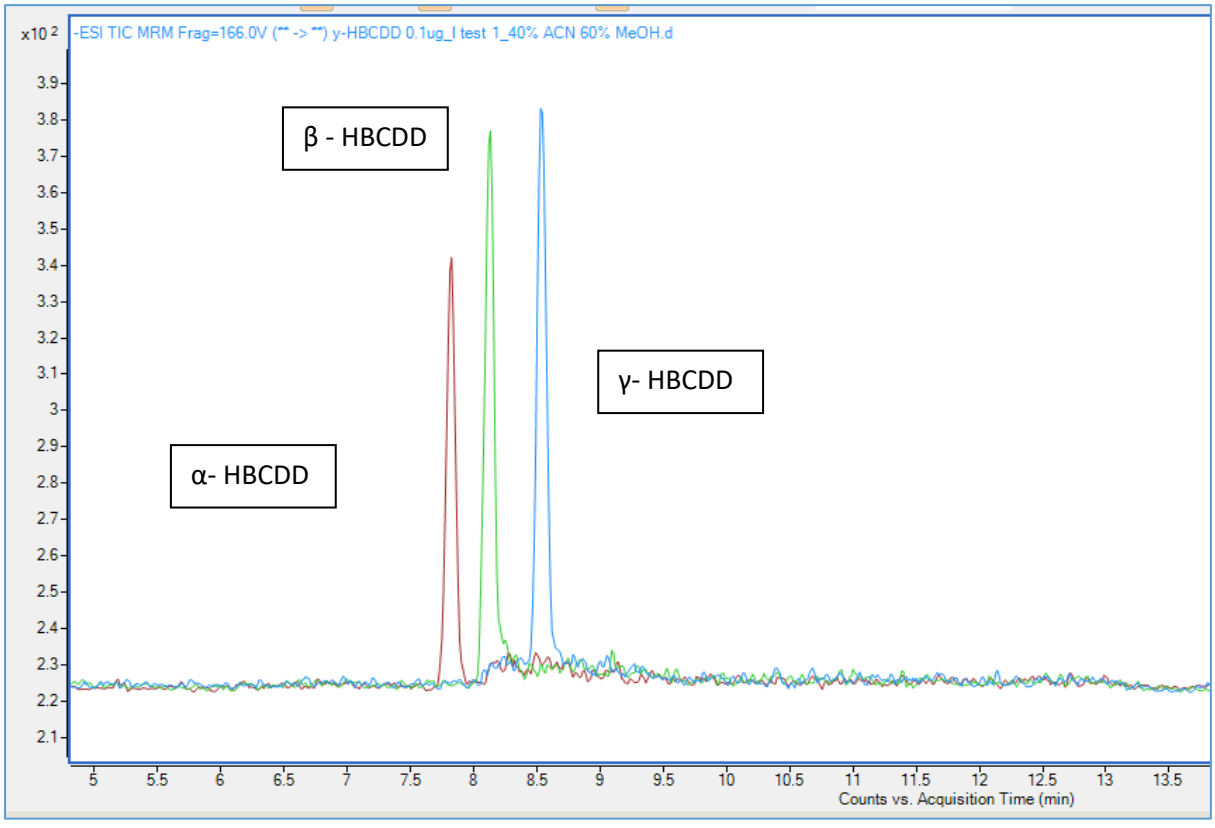


Figure L.7: 30% ACN and 70% MeOH as mobile phase B

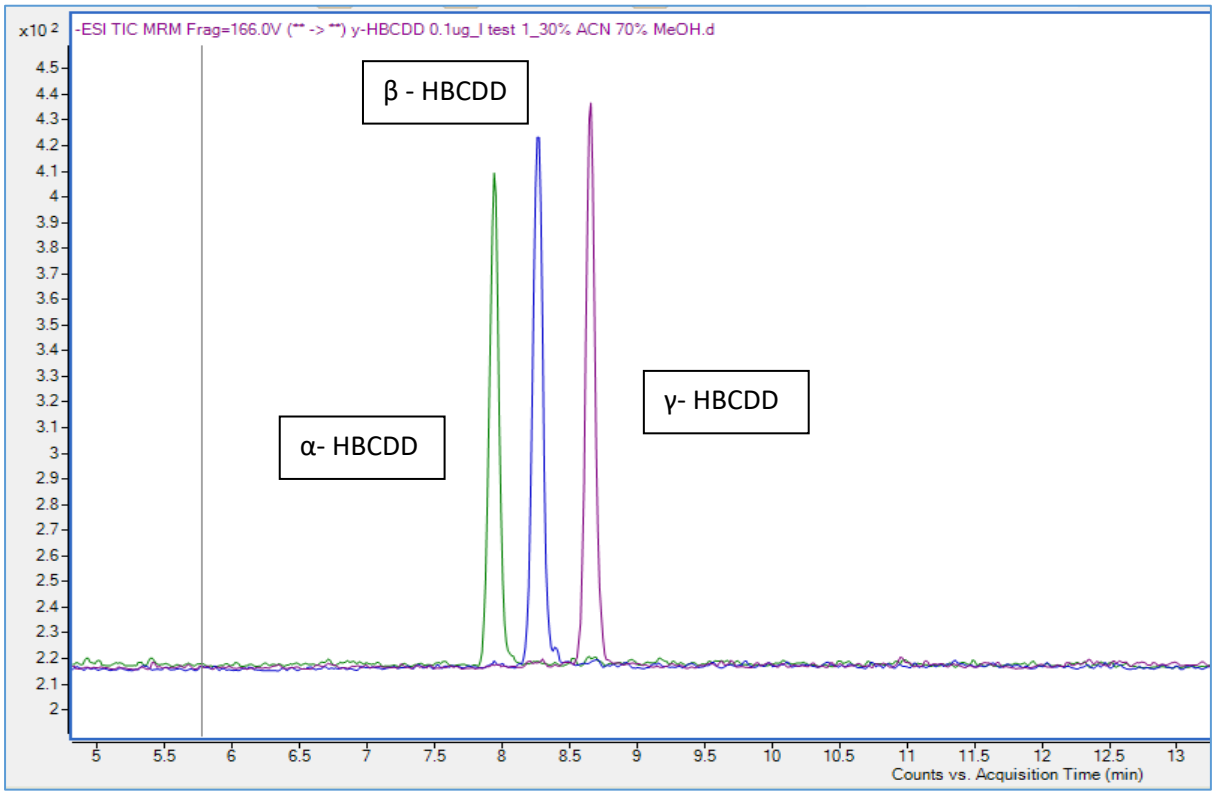


Figure L.8: 20% ACN and 80% MeOH as mobile phase B

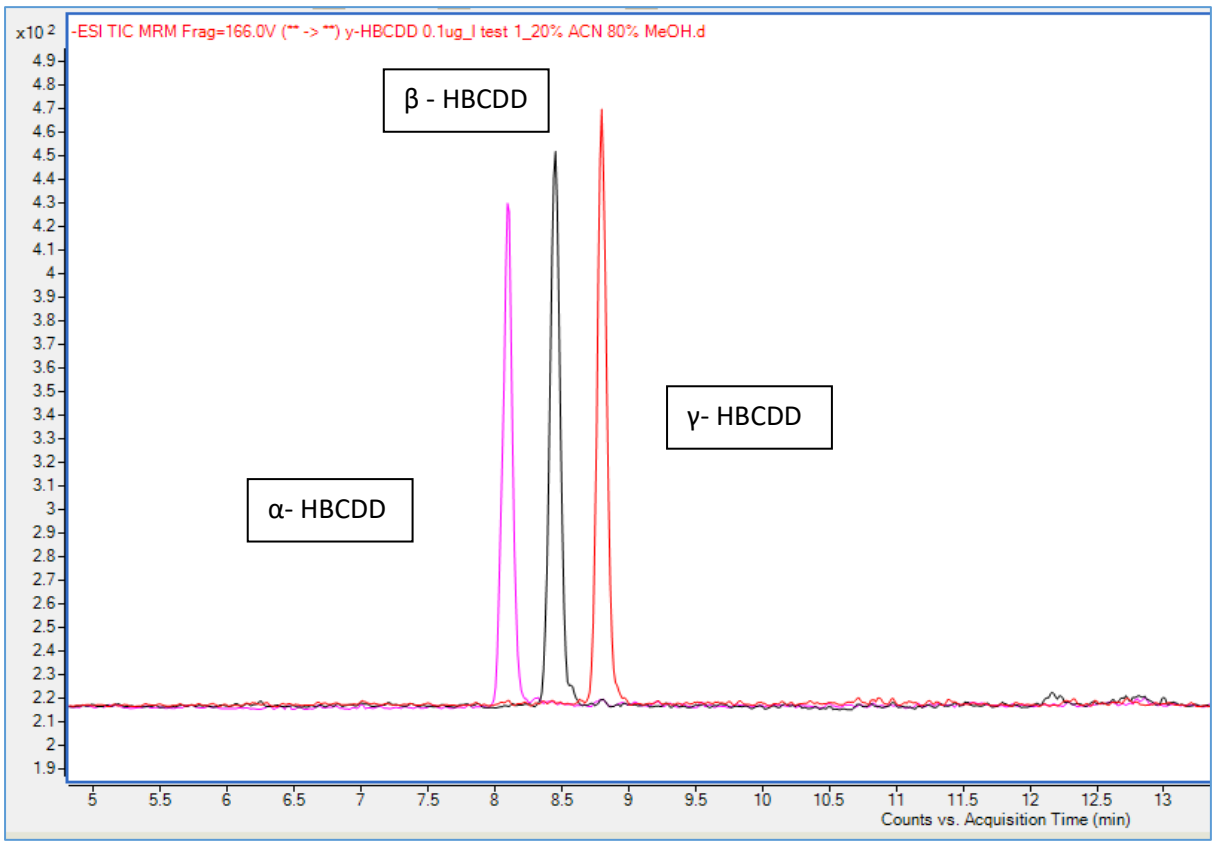
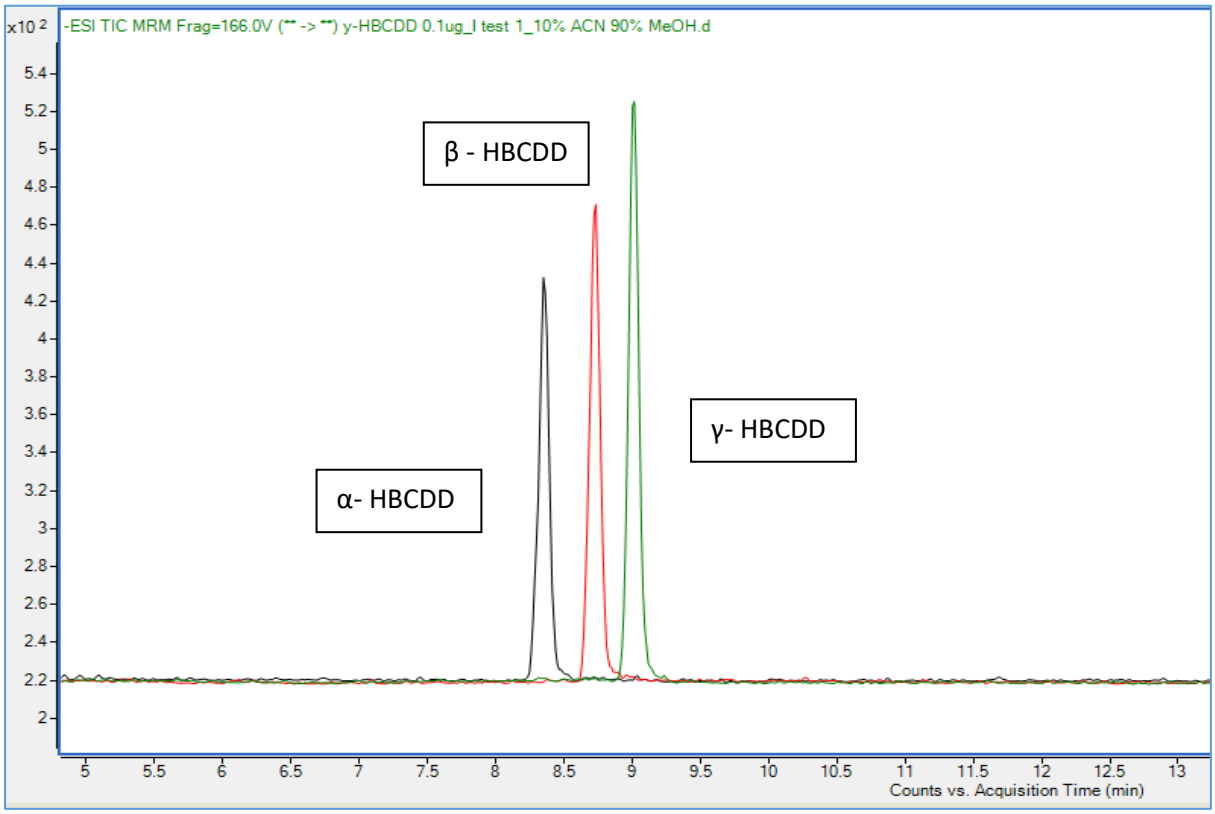


Figure L.9: 10% ACN and 90% MeOH as mobile phase B



Appendix M: Overlaid chromatograms of triplicate samples for each dSPE clean up kit

Figure M.1: 'General Fruits and Vegetables' dSPE

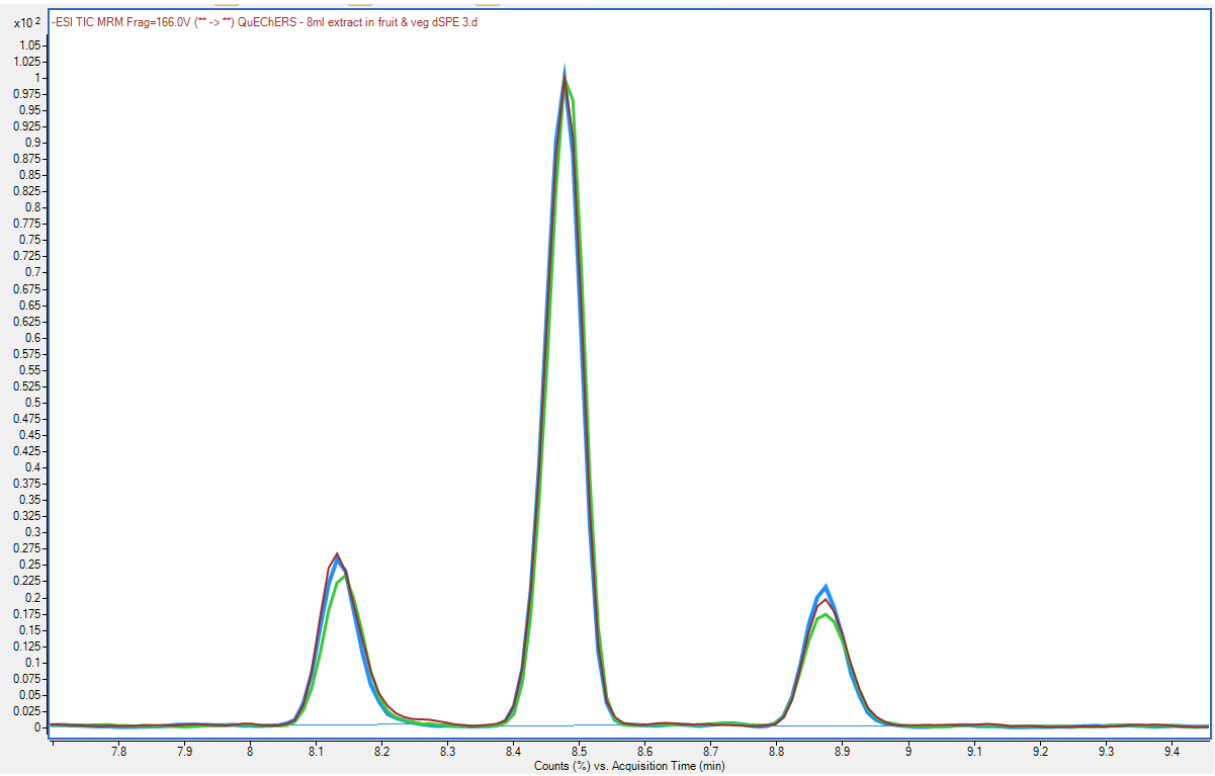


Figure M.2: 'Fruits and Vegetables with Fats and Waxes' dSPE

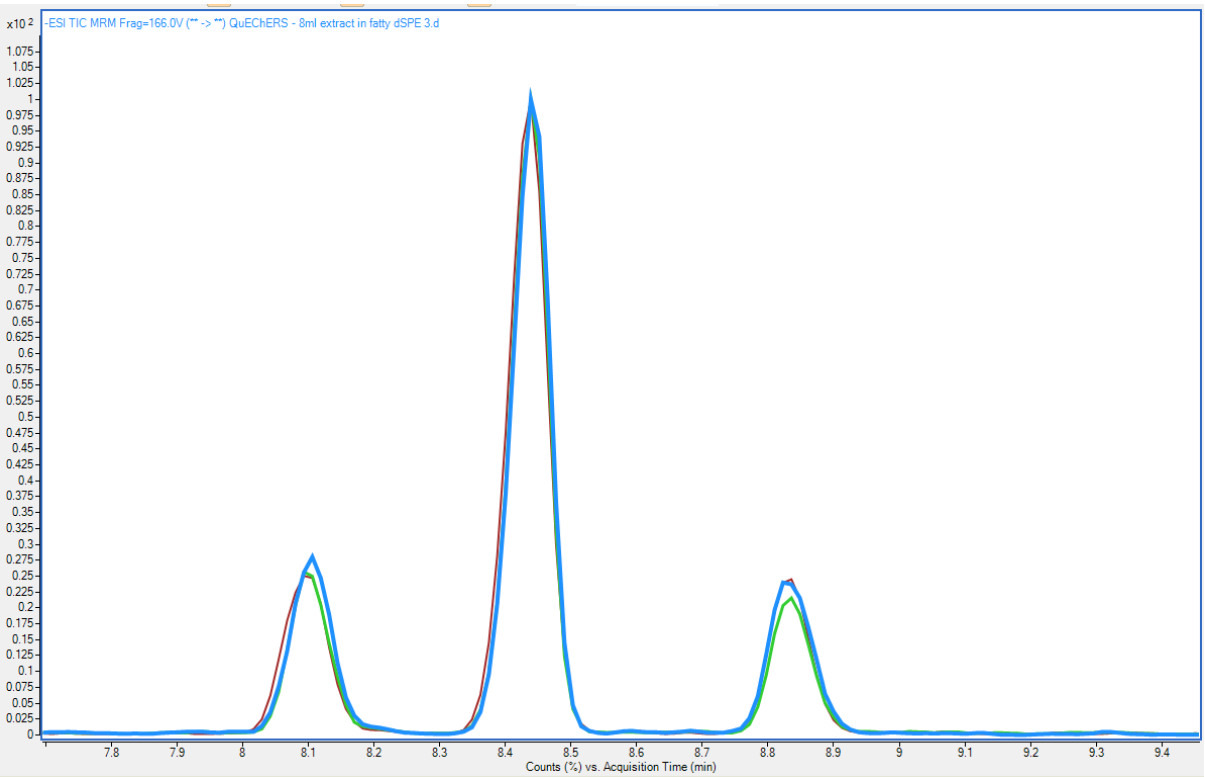
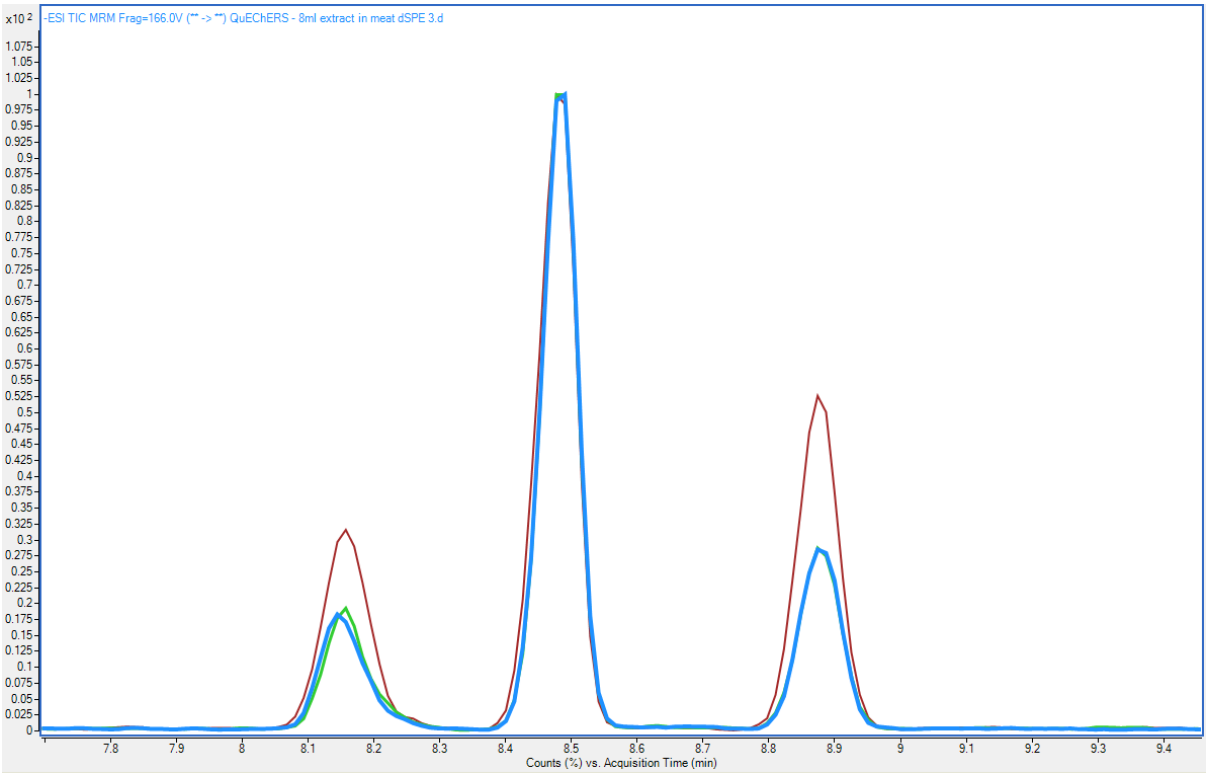


Figure M.3: 'Drug Residues in Meat' dSPE



Appendix N: Chromatograms of QuEChERS extraction procedure with modified volumes of water and acetonitrile

Figure N.1: 5 mL of water and 10 mL of acetonitrile in salt extraction step

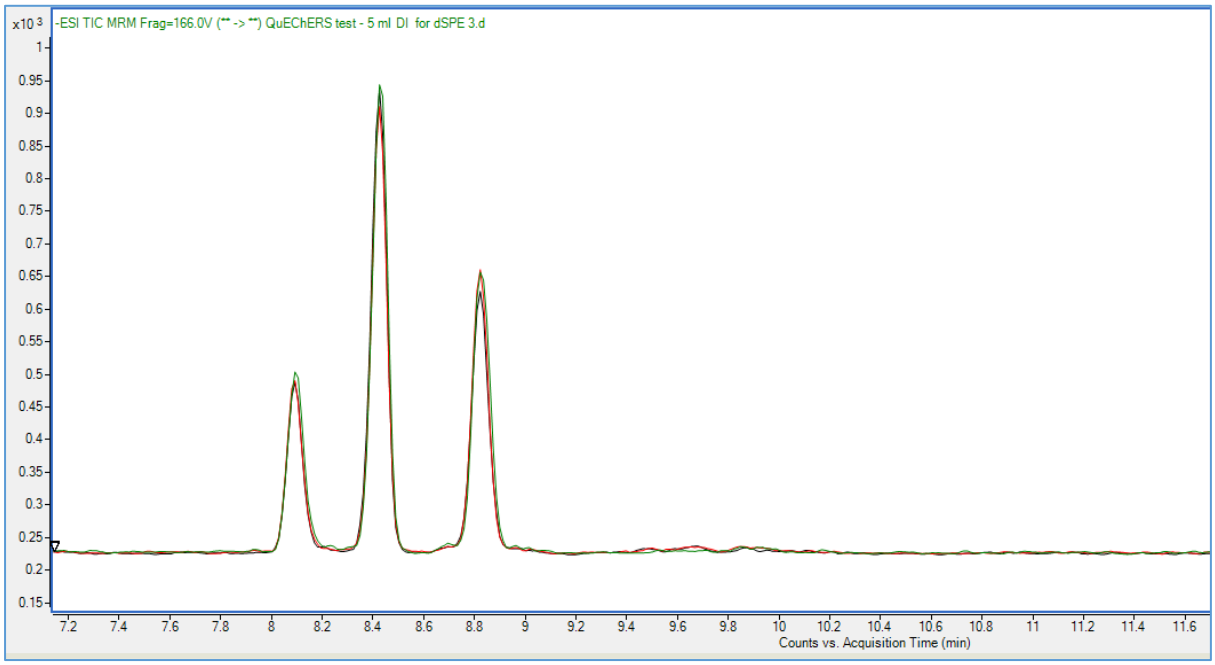
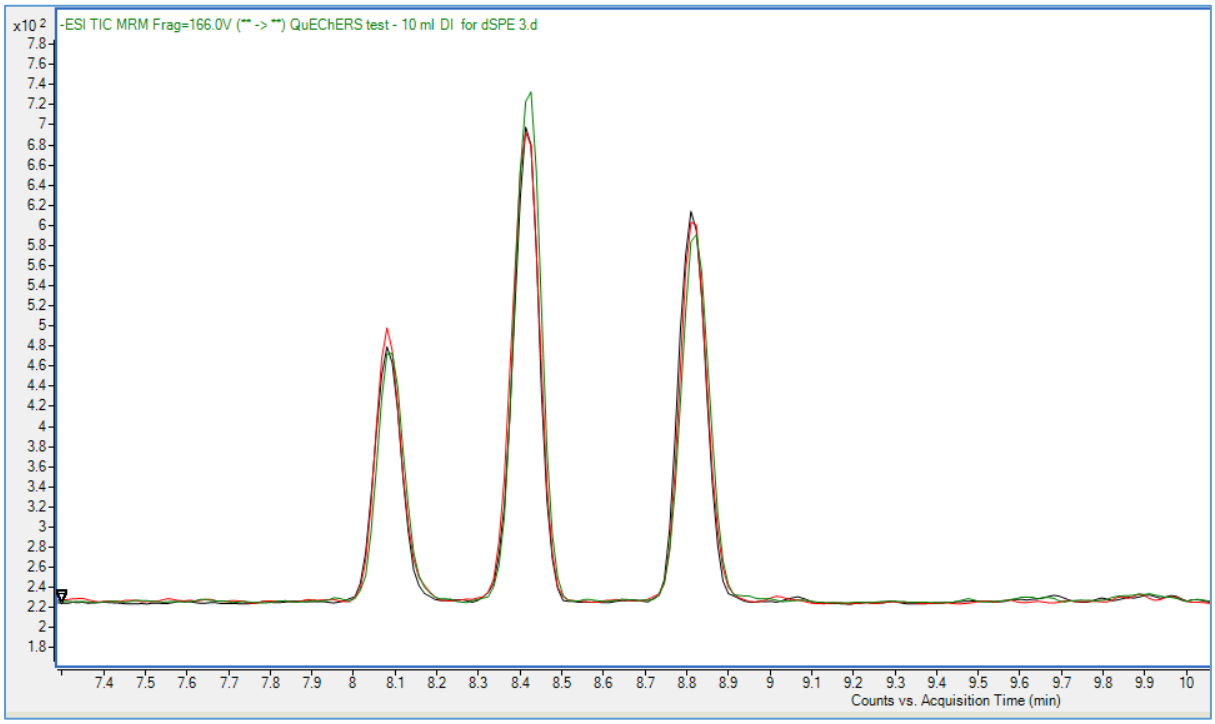


Figure N.2: 10 mL of water and 15 mL of ACN in salt extraction step



Appendix O: Chromatograms showing HBCDD recovery with different volumes of water added to salt extraction step and <u>no</u> clean-up step

Figure O.1: 5 mL of water added

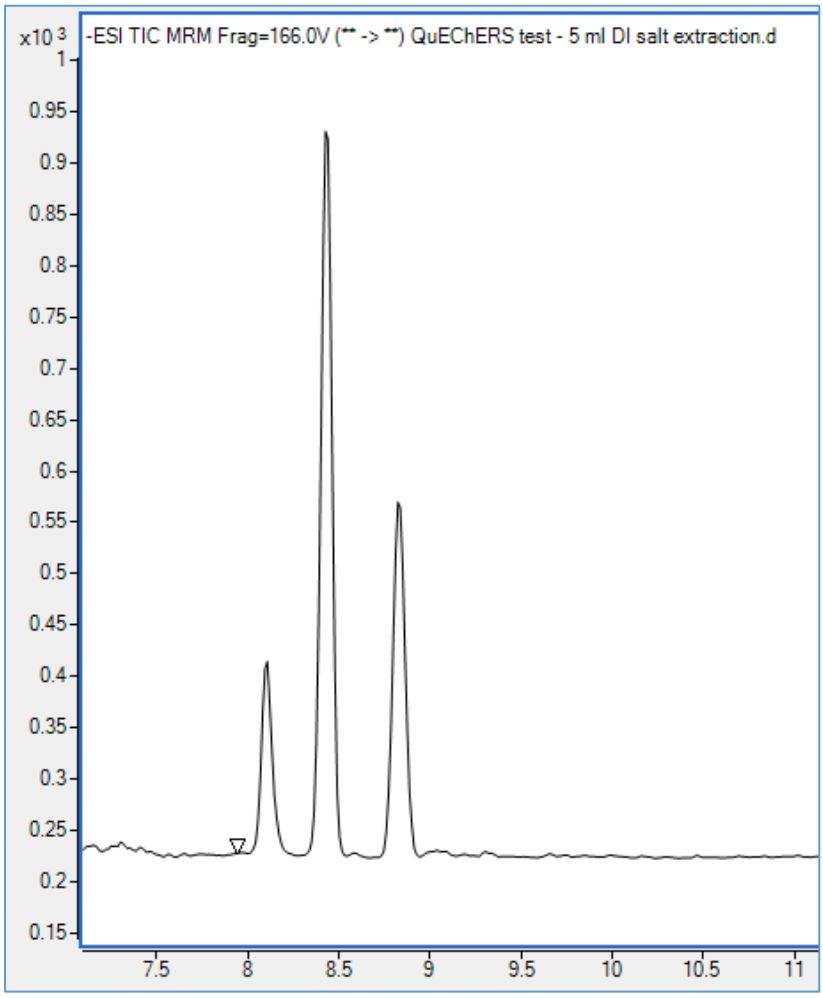
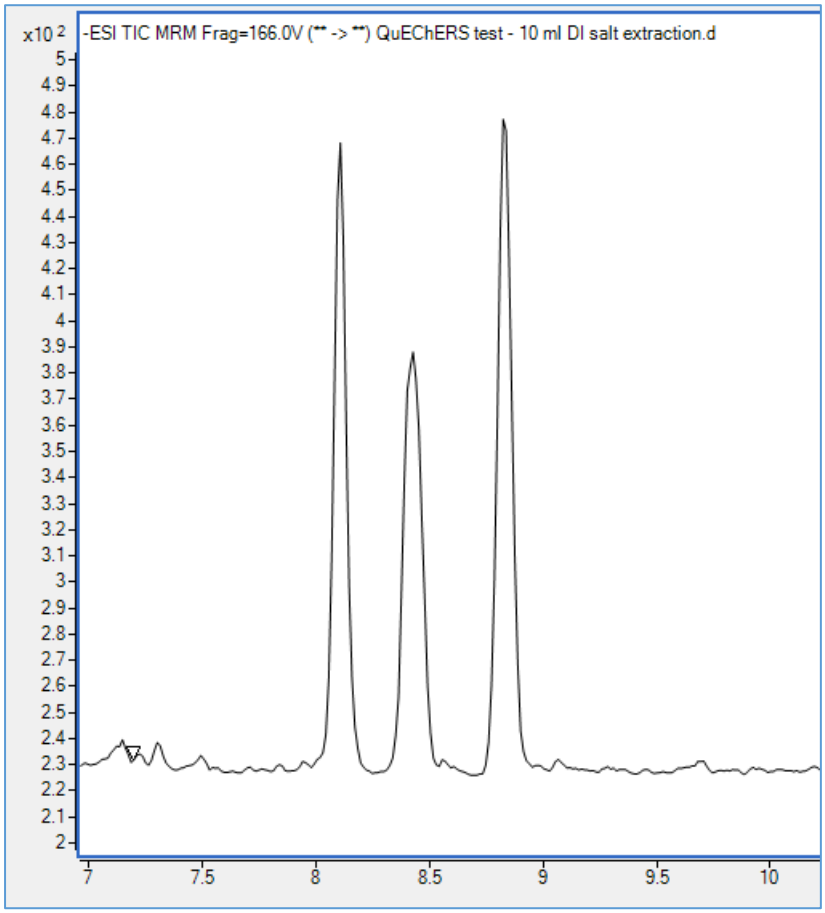


Figure O.2: 10 mL of water added



Appendix P: Chromatogram showing different volumes of extraction solvent taken for clean-up procedure based on volume of water and acetonitrile added in the extraction stage

Figure P.1: 4 mL of extract taken from extraction solvents of 10 mL addition of water and 15 mL ACN used in extraction step

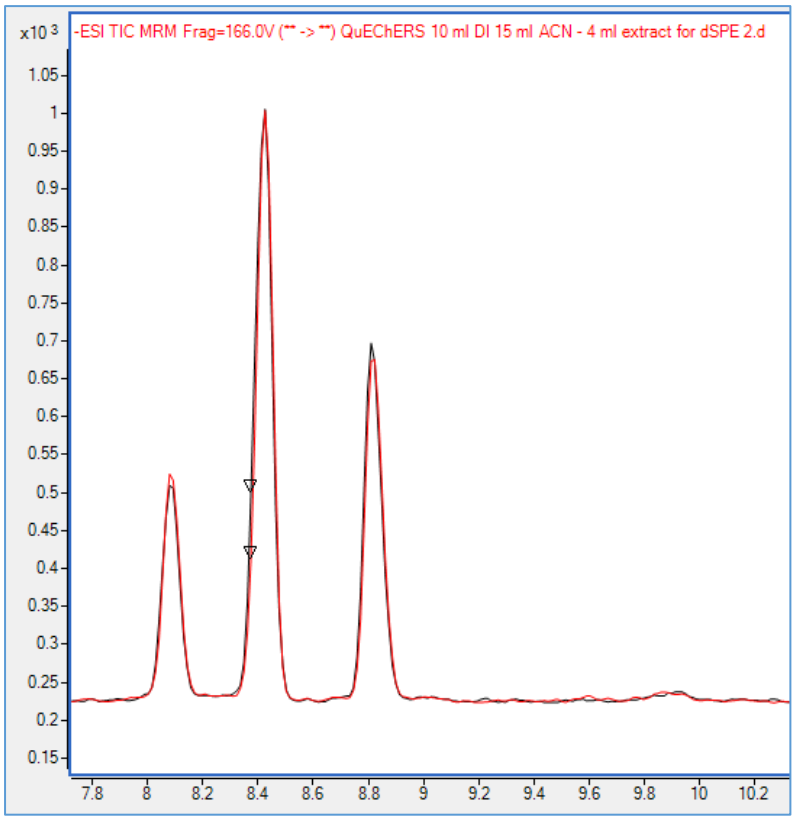
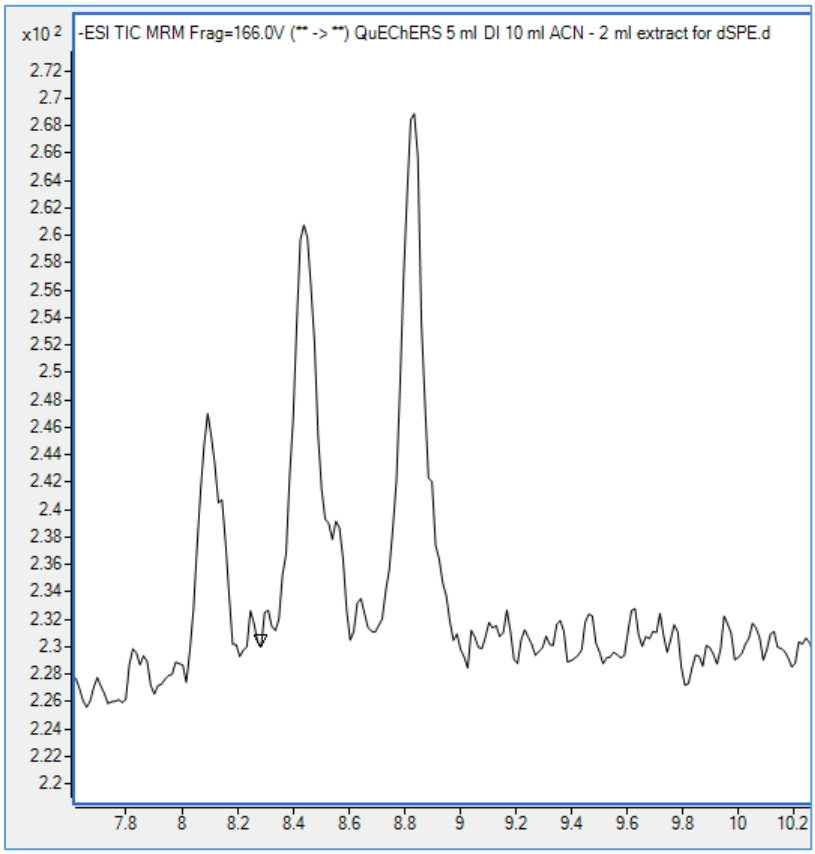


Figure P.2: 2 mL of extract taken from 5 mL addition of water and 10 mL ACN used in extraction step



Appendix Q: Illustration of the QuEChERS procedure with alternative volumes

Figure Q.1