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An abstract of
A thesis submitted to the Faculty of the
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Abstract

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After the toxicity of polybrominated biphenyls or PBBs became evident, they were replaced by a similar compound, whose toxic effects on the human body are not fully known. The ubiquitous and rising presence of polybrominated diphenyl ethers (PBDEs) in the environment has given rise to a variety of hypotheses of their role in environmentally-related disease. Like many other persistent organic pollutants, their lipophilic nature has caused them to bioaccumulate and biomagnify, and because of their continued use, they have been detected in similar concentrations among all age groups. In order to understand the association between exposure to PBDEs and neurologic disease, we must first be able to quantify exposure in the target tissue – the brain, and until now, no method existed for doing so. Using the information available in the existing literature, we have developed a novel method for analyzing PBDEs in human brain tissue. The analytic complications encountered when using lipid-rich matrices were overcome by employing a careful, selective extraction procedure/cleanup in addition to the use of tandem mass spectrometry (MS/MS) analytical techniques. The data collected from this study demonstrates that the newly developed method can successfully quantify PBDEs in brain tissue and was confirmed by the quantification of PBDEs in the archived brain samples. This novel method will provide a basis with which to work for future studies and will enable the progression of epidemiological research to study the association between exposure to PBDEs and their potential relation to neurodegenerative disease.

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Table of Contents

1. Introduction

Polybrominated diphenyl ethers (PBDEs) belong to a class of compounds known as brominated flame retardants (BFRs). Currently, there are more than 175 chemicals classified as flame retardants. The four major groups are inorganic, organohalogenated, organophosphorus and nitrogen-based flame retardants, with the organohalogenated type accounting for 25% of annual production.[1] Part of the broader class of organohalogenated compounds, BFRs have been used in a wide array of applications, such as the manufacturing of electrical transformers[2]. BFRs are produced synthetically in 70 variant forms and are used in a variety of consumer products, several of which are produced in large quantities.[3] We are surrounded by a wide variety of BFR polymers in everything from clothing and furniture to vehicles and electronics. Since most of these polymers are petroleum-based and flammable, flame retardants are applied to combustible materials, such as plastics, wood, paper and textiles in order to meet safety regulations.[1] Usage of BFRs is mainly confined to the electronics and textile industries in printed circuit boards, plastic covers, cables, television sets, radios, carpets, paints, upholstery, and kitchen and office appliances. They are also added to construction materials, and transportation vehicles to protect those who may be caught in a burning building or vehicle.[4]

Based on the mode of incorporation of the compound into the polymer, BFRs can be further classified into three subgroups – brominated monomers, reactive or additive. Brominated monomers, such as styrene, are used in the production of polymers. Reactive compounds, such as tetrabromobisphenol A (TBBPA) are chemically bound into the plastics. Additive flame retardants, such as PBDEs and hexabromocyclododecane, are blended with the polymers but are not covalently bound in them.[1] PBDEs became a popular replacement for PBBs (polybrominated biphenyls) once the environmental consequences of their use became evident. However, PBDEs were not tested for human or environmental toxicity prior to their introduction into manufactured products. BFRs are currently the largest flame retardant market group, with TBBPA being the

highest volume BFR on the market. PBDEs follow them being the second highest because of their low cost of production and high performance efficiency.[1,4]

The efficiency of PBDEs as flame retardants is attributable to its chemical structure. The 209 different PBDE congeners are classified and named by the degree of bromination. They are similar in structure to PBBs and polychlorinated biphenyls (PCBs) except instead of having two benzene rings directly connected to each other (and halogenated with chlorine atoms in the case of PCBs), PBDEs consist of two benzene rings attached by an oxygen atom (hence, the name "ether") and are halogenated with bromine atoms. PBDEs can range from having two bromine atoms (dibromo-) to ten bromine atoms (decabromo-) with each bromination level called a homolog. The naming and numbering scheme is also based upon the system used to name PCBs proposed by Ballschmiter and Zell, 1980.[5]

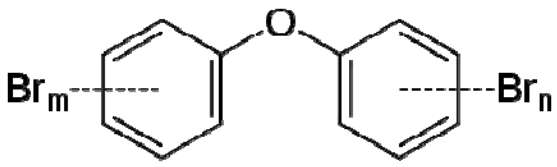

Figure 1. PBDE Molecular Structure

PBDEs are marketed with trade names and are rarely sold as individual congeners. In 2001, the world demand for PBDEs was 67,390 metric tons with deca-BDE constituting 83%, octa-BDE constituting 6%, and penta-BDE constituting 11%.[6] The degree of bromination not only determines its use in industry but also the danger it poses to humans and the environment. Congeners that average one to five bromine atoms per molecule are considered more dangerous because they bioaccumulate more efficiently. They are smaller in size and can get into tight spaces between and within cells in the body, including crossing the blood-brain barrier whose job is to prevent certain molecules from entering the brain where they accumulate in fatty tissue[7]. Lower-brominated PBDEs (one to five bromines) have been shown to affect hormone levels in the thyroid gland. PBDEs with four to six bromines (*e.g.*, BDE-47, 153, and 154) tend to be bioavailable and form metabolites similar in size and structure to thyroxin (T4). The mechanism of toxic action is not known, but it is thought that PBDEs mimic the T4 hormones, alter hormone levels and cause endocrine disruption, which can lead to developmental changes in newborns.

Studies have also linked lower-brominated PBDEs to reproductive and neurological risks at or above certain concentrations.[8] Higher-brominated PBDEs (greater than 5 bromines) have been found in lipid tissues in both humans and animals, but deca-BDEs typically breakdown into lower-brominated forms prior to human exposure[9]. To better understand how to mitigate exposures thus reducing any associated health effects, it is important to know the sources of exposure.

Animals are exposed through the environment from waste dumped into rivers and oceans by manufacturers and from consumer product waste known as e-waste. Commonly, people discard old electronics the same way they discard their trash. Computer parts and furniture often end up in landfills where they are in contact with the soil and contaminate the surrounding area. Since PBDEs are additive flame retardants, they are not bound by any material and are not anchored to the object it is intended to protect. This allows the PBDEs to leach out of the material and become bioavailable from air, soil and water.[10] Because of their low chemical reactivity and extreme hydrophobicity, PBDEs are persistent and bioaccumulate.[11]

When PBDEs are ingested via water or soil consumption or inhaled via air as re-suspended dust particles, their hydrophobic nature causes them to accumulate in lipid-rich tissues. Unless that fat is being shed constantly, PBDEs are not readily eliminated by the body. This is what categorizes PBDEs as a persistent organic pollutant and adds to the biomagnification potential of PBDEs. Biomagnification and bioaccumulation are not the same. Bioaccumulation is when a compound is stored in an animal's tissue and builds up over time whereas biomagnification is when the amount of that compound increases as it moves up the food chain. For example, a fish living near a landfill that feeds on organisms in the sediment will accumulate PBDEs in its fatty tissue. When a bigger fish eats that fish, it accumulates the PBDEs from that fish in addition to any PBDEs already in its body. This compounding effect continues up the food chain, to sharks, polar bears, and even humans.

A person can be exposed to PBDEs from non-dietary sources as well since PBDEs are used in everyday products that we touch, such as electronics, cars, and furniture, to name a few. The leaching of these compounds allows them to enter the air and mix with other particles floating around creating dust particles. In addition to dermal exposure from touching dust, we inhale dust in the air regularly. Children are typically at higher risk for non-dietary ingestion and dermal exposure since they are usually crawling on the ground or putting objects and their hands in their mouths. Consequently, children's exposure has become an area of great interest in current research initiatives.

Research has shown that occupational exposures can contribute greatly to the PBDE body burden in addition to dust inhalation and the ingestion of contaminated food and water. A 1999 study of Swedish hospital cleaners, computer clerks and electronics-dismantling workers showed higher levels of five particular congeners that are believed to be biologically relevant. These congeners were BDE-47, 153, 154, 183 and 209. Researchers did not previously believe that a deca-BDE, such as BDE-209, could bioaccumulate because of its large molecular size and weight, however this study revealed that all bromination levels of BDEs are able to be taken up by the body and have the potential to do damage.[12]

As previously mentioned, lower-brominated PBDEs have the highest potential to cause adverse health outcomes. This is likely due to the size of these compounds being similar to biologically-relevant molecules. Penta-BDE mixes were removed from the market[9] since they were found to mimic hormone activity, disrupt the normal levels of thyroid hormones, and cause endocrine disruption. This can result in delayed reproductive development and can affect the liver by causing an increase in liver weight and incidence of tumors via induction of liver enzymes. PBDEs also cause estrogenic effects through the inhibition of estrogen sulfotransferase.[4]

The carcinogenic potential of PBDEs has also been studied. Animal studies have shown that mice that ate food contaminated with PBDEs developed liver tumors. This significant finding led the U.S. Environmental Protection Agency (USEPA) to classify PBDEs as possible human carcinogens.[6] A newly emerging concern about the neurodegenerative effects of PBDEs has led to an increase in this area of research. For instance, Mariussen and Fonnum reported inhibition of vesicular uptake of dopamine at low micro-molar concentrations of a PBDE mixture (DE-71). The alteration of dopamine homeostasis and the generation of reactive oxygen species have been suggested to play a central role in the pathogenesis associated with Parkinson's disease (PD). Preliminary research has shown that exposure to PBDEs in rats shows a dose response decrease in dopaminergic neurons.[13] Still, the mechanism of how neurologic diseases, such as PD and Alzheimer's disease (AD), develop is not well understood. Many researchers propose that genetic factors play a role, but the known pathology comes from greatly reduced activity of dopamine-secreting cells due to cell death in the pars compacta region of the *substantia nigra* in the brain.[14] Other speculation suggests that certain PBDE congeners are able to pass the blood brain barrier and accumulate in this region of the brain. A proposed mechanism of toxicity in PD is the accumulation of insoluble proteins that encroach into the *substantia nigra*, causing cell death.[15] If PBDEs are able to accumulate in a similar fashion, it could produce the same neurodegenerative effects. The mechanism of this toxicity is unknown, and thus, remains an active area of research.

With new information about the toxic effects of PBDEs emerging daily, the dangers of exposure have become increasingly real. PBDEs have been found in a wide assortment of biota, ranging from fish and Arctic wildlife to humans.[12] Some of the most significant concerns today are the environmental and biological pervasiveness of these compounds such that they are appearing in important nutritive food sources for infants such as human breast milk. A study of Swedish women showed that the concentrations of PBDEs in breast milk have increased since 1972[11] although they have declined sharply since regulatory actions to reduce PBDE use were put

into place. Regardless, implications from this study raise concerns about children's exposure to PBDEs from breast milk. With information about the reproductive and endocrine disrupting effects coupled with the carcinogenicity and neurotoxicity of PBDEs becoming more apparent each day, concern for the health of our children is highly warranted.

Large-scale biomonitoring studies of chemicals in human matrices have provided snapshots of human exposure to a wide array of chemicals, some of which are manufactured in concentrated geographical regions where the natural resources are located. PBDEs, for example, are manufactured largely in the southern region of Arkansas, home to the Albemarle and Great Lakes Chemical Corporations. The brine wells in this region are rich in bromine, and the Great Lakes Chemical Corporation is solely responsible for the production of 40 million pounds of methyl bromide annually. Predictably, its bromine business has made the company the number one polluter in Arkansas based on the 1994 Toxic Release Inventory data.[16] The Albemarle and Great Lakes Chemical Corporations, along with the Dead Sea Bromine Corporation, make up an oligopoly in the bromine product market. In 2001, the United States used about 149 million pounds of PBDEs, half the world's total.[17]

The large production and consumption of these hazardous chemicals puts Americans at serious risk for adverse health outcomes from a chemical we believe to be harmful. Many of these chemicals, however, are not tested before being introduced into the market. As information about the toxic effects of chemicals in existing use come out, new chemicals are created to replace them, without testing. In some cases, we find that chemicals once banned for a specific use find their way back into the marketplace as chemicals with new uses.[18] Similar to the European Union, resources should be devoted to testing new chemicals for toxicity before they are put on the market. If this policy is not adopted, the cycle of discovering that the chemicals we currently use are toxic and replacing them with untested chemicals will continue proving more harm than good.

Fully understanding a chemical before allowing its release into the market is critical to environmental and human safety. As an environmental health public health researcher, I help to characterize these chemicals to elucidate further information about their overall safety. For my thesis, I investigated PBDEs in post mortem human brain tissue to better understand their role in the progression of neurodegenerative disease. This is the first time that this matrix has been tested for PBDEs. As such, I plan to complete this study by extracting PBDEs from brain tissue via solid-liquid extraction followed by solid-phase extraction (SPE) cleanup and analyze the samples using gas chromatography with tandem mass spectrometry (GC-MS/MS). I have analyzed 44 tissue samples that were obtained from the Emory Brain Bank – 15 from patients diagnosed with Parkinson's disease, 15 from patients diagnosed with Alzheimer's, and 14 control samples.

The specific aims of my research are to: (1) develop and validate an analytical method for measuring PBDEs in human brain tissue; and (2) measure PBDE concentrations in human brain tissue of decedents with neurodegenerative disease and in those with no known neurological disease. I hypothesize that PBDEs will be measureable in all brain tissue samples but that PD and AD patients will have higher levels of PBDEs than in control patients. This research thesis will add to the limited literature base regarding the role that PBDEs play in the pathogenesis of Parkinson's and other neurological diseases.

2. Methods

2.1 Basis of Method

Since no method has been developed for the extraction of PBDEs in brain tissue, I had to use the available literature on extraction of PCBs from mice brain, a comparable matrix because of its similarity in fat content. This would serve as a starting point for developing a new method with brain as the matrix of interest. During the beginning stages of method development, there were many changes. All of the versions of the method contain three stages: Liquid extraction,

cleanup, and solid phase extraction (SPE). Throughout the method development stage of this study, many variables were changed or altered to optimize method parameters including sample weight, solvent volumes, temperature and pressure profiles, type of test tube used, and the method of reconstitution.

2.2 Standard Preparation

The method was developed based on the PCB literature and it was decided that based up the highest level of on-column quantification of 2 pg achievable with our instrumentation, the final extract (in 100μL) was to have 25 pg/μL concentration. This method called for 50 mg of brain tissue to be spiked with 1 ng of each PBDE congener of interest for internal standard. In addition to having internal standard, two quality control (QC) standards were created, a low one that would equate to 1 ng PBDE per brain sample and a high one that would equate to 3 ng PBDE per brain sample. The spiking volume of these standards was set at 100 μL, which required a 10 ng/mL solution and a 30 ng/mL solution, respectively, for a 50 mg sample of brain tissue. These standards were created in hexane because hexane is a very non-polar solvent and is readily able to extract non-polar compounds such as PBDEs. For the remainder of this section I will discuss the steps of the final method.

2.3 Equipment and Materials

The final method for extracting PBDEs from brain tissue required chemical fume hood space to work in, solvents for extraction and other general laboratory equipment. Special equipment needed included a tissue homogenizer, sonicator, centrifuge, Turbovap evaporator (Zymark Corporation, Framingham, MA) and Restek (Bellefonte, PA) 3mL SPE cartridges with a 500mg Florisil sorbent bed. Solvents included acetone, hexane, and toluene which were purchased from the chemistry department at Emory University (Atlanta, GA). Other equipment

needed included one set of 16x100mm borosilicate glass test tubes and three sets of 15-mL conical centrifuge tubes (per extraction), pipettes, beakers, protective equipment like gloves, goggles, and lab coats, and a scale for weighing tissue aliquots.

2.4 Procedure

The first step in the extraction procedure was to accurately weigh ~50 mg of brain tissue into a 16x100 mm glass tube with 100 µl of internal standard (ISTD) which consisted of ^{13}C-labeled analogues of each PBDE congener. ISTD was added to provide a standard unit of measurement of PBDEs at a particular concentration so that the results may be compared relative to this standard for accuracy and automatic extraction recovery correction. Next, 0.5 mL of acetone and 0.5 mL of hexane was added to the tube. This amount of solvent was enough to completely submerge the 50 mg sample and allow for complete homogenization. The sample was ground using the tissue grinder until completely homogenized in solution and the content of the tube was transferred to a 15 mL conical centrifuge tube. The 16x100 mm tube was rinsed with an additional 1.0 mL hexane and 1.0 mL acetone in order to transfer any remaining tissue. Both acetone and hexane were used because they are miscible yet have different chemical properties that allowed for maximum recoveries. Next, the sample was sonicated for 15 min and then vortex mixed for 2 minutes. Once it had been transferred, the tube was centrifuged for 10 minutes at 2500 rpm and 4°C. After centrifuging, the supernatant of the tube was transferred to another clean labeled 15 mL conical test tube that was pre-weighed and recorded so that the residue left after evaporation could be weighed and measured. During this transfer, the supernatant needed to be filtered through a 20 µm pore frit to remove particulates and proteins. This extraction procedure was repeated four additional times before moving on to the cleanup stage.

After the liquid-liquid extraction had been performed 4 times, the samples were evaporated at 45°C using 15psi of nitrogen until they were dry and only residue was left in the

tube. Each sample was reconstituted using 0.5ml hexane and 0.5ml acetone and was then vortex mixed to get the residue back into solution. At this point, a vacuum manifold was prepared for SPE. Restek 500mg (6cc) Florisil cartridges were used to remove biogenic materials, especially lipids, from the samples so that only PBDEs would pass through in solution. But before the samples were loaded into the cartridge, the cartridges required conditioning. Each cartridge was conditioned using 1 mL of hexane in order to wet the sorbent and avoid creating channels in the sorbent. Once the cartridges were conditioned, a new set of clean labeled test tubes was inserted into the collection chamber of the manifold. Each sample was loaded into its respective cartridge and the sample was allowed to pass through the Florisil column bed. After loading the samples, cartridges were eluted with 2 mL of hexane, 5 times. At this point only hexane was used because we only wanted PBDEs to pass through the cartridge. After elution, the samples were evaporated again, but this time they were not taken to dryness. Instead, the samples were brought down to approximately 1 mL volume (~10min @35°C, 10 psi nitrogen) and 100 μL of toluene was added for solvent exchange since toluene is a more suitable GC injection solvent than hexane. The samples were then evaporated down to approximately 100 μL and at this point, all of the hexane would have evaporated off due to its high volatility, leaving only toluene. We did not want to bring the samples to dryness again because there was a chance we may have lost some compounds during evaporation because there are fewer lipids to retain them in the tubes. The remaining 100 μL was then transferred to GC vials for analysis.

The method of analysis involved the use of a GC-MS/MS to selectively identify and quantify individual congeners by first separating the congeners in time then by mass. The GC utilizes a capillary column with the column's dimensions (length, width, etc) dictating the temporal separation of the congeners. The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute from the column at different

times (retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio. The temperature profile and settings for the GC separation were received from Cambridge Isotope Laboratories, the same company that makes the standards used in this study. Details of the GC-MS/MS method are given in Appendix A.

2.5 Method Validation

To ensure the method was working, a series of validation and recovery studies were done. To determine the percent recovery, samples were spiked with native standard at different times throughout the extraction procedure. With a known concentration being spiked into the samples at different points in the procedure, we were able to determine if any step in the process was causing us to lose the compounds of interest. Samples were spiked at three different points in the procedure, once after the sample had been homogenized but before sonication, once after the sample had been reconstituted but before it was passed through the Florisil column, and once after the elution with hexane, but before the final evaporation and solvent exchange with toluene. The first spiking point would determine if compounds were being lost prior to or during the first evaporation. The second spiking point would determine if compounds were being lost during the elution through the Florisil column. The third spiking point would determine if compounds were being lost during the second evaporation. To test this, a total of 18 samples were extracted, nine samples at the low standard concentration (1 ng/sample) and nine at the high standard concentration (3 ng/sample). Three of each nine were assigned to the first spiking point, three to the mid-spiking point, and three to the last spiking point. Once we had determined that compounds were not being appreciably lost during each step of the extraction procedure, a total recovery study was done using triplicate samples at both low and high standard concentrations. The result of this study can be seen in Figure 2.

Precision and accuracy were measured through a series of validation studies that were done in succession and analyzed similarly to avoid any variation in the instrumentation that could have influenced the analysis. Five extractions, each consisting of one brain sample, spiked only with internal standard, three QC low samples, spiked with 1 ng PBDEs/sample standard in addition to internal standard, and three QC high samples, spiked with 3 ng PBDEs/sample standard in addition to internal standard, were completed within five consecutive days. The results of this five-day validation study were used to calculate the limit of quantification (LOQ) as well as precision and accuracy. Both within-day and among-day relative standard deviations (RSDs) were calculated. Accuracy, although typically defined based upon a reference material that was not available for PBDE analysis, was defined as the percentage of agreement between the spiked value and the quantified value at different concentrations. According to the Food and Drug Administration guidelines for pharmaceutical studies, accuracies of 80-120% and RSDs <20% are deemed acceptable for a quantification method for biological matrices. The limits of detection (LOD) were defined as the concentration at which the signal-to-noise ratio (S/N) of the observed signal was > 3. These values were extrapolated from the S/N of the lowest standard with the S/N closest to 3.[19] The LOQs were defined as the lowest standard with a S/N ratio > 3, typically the standard from which the LODs were derived. However, it is important to note that these LODs and LOQs were are dynamic values and may change based upon matrix variations, instrumental performance, analyst performance and other parameters.

2.6 Statistical Analysis

Microsoft Excel (Redmond, WA) software (version 12.0) with the Data Analysis Toolpak add-on was used to analyze the data. Within, among and total precision of analyses were calculated using the quality controls samples. Shewart plots, typically used for quality control in clinical laboratories, were constructed to visualize the variation in quality control samples over time. Distribution percentiles, geometric mean, arithmetic mean, standard deviation and standard

error of PBDE concentrations in human brain samples were calculated. Histograms were created to depict the overall distribution of the data. The distributions were tested for normality using SPSS software (Version 18 Armonk, NY). Although PBDE concentrations were evaluated based upon disease status, the comparisons were just preliminary and tenuous as the values must be adjusted based upon the lipid content of the brain. Determining the lipid content of non-standard matrices is difficult and we are still developing procedures for this assay.

2.7 PBDE Instrumental Method Details GC-MS/MS

The method used for the analysis of PBDEs-47, 99, 100, 153, and was developed in-house in the Barr/Ryan Laboratory. The validation results indicate good precision, accuracy and limits of detection. Chromatographic separation was performed by Agilent 7890A Gas Chromatography (GC) with tandem mass spectrometry (Agilent Technologies, Waldbronn, Germany). The GC consisted of a temperature-stable column compartment. The GC and MS modules were programmed and controlled using Mass Hunter Software version B.03.01 (B2065) (Agilent Technologies, Waldbronn, Germany). PBDEs were analyzed by GC-QQQ-MS/MS (EI, -70eV). Analyses were carried out using a 7890A GC coupled to a 7000B MS (Agilent Technologies, Waldbronn, Germany). The system was fitted with a deactivated silica guard column (0.250 mm internal diameter (ID)) (Agilent Technologies, Santa Clara, CA USA) connected to a HP-5MS analytical column (15m x 0.250 ID x 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA USA). It was operated in pulsed splitless mode (24.656 PSI, 0.85 min) with an injector temperature of 250°C). The helium carrier gas flow rate was 1.8 mL/min and the oven temperature program was as follows: 100°C (0.1 min), 250°C (45°C/min), 275°C (5°C/min), and 315°C (45°C/min) held for 4 min. The interface, source, and quadropole temperatures were set to 315°C, 315°C, and 150°C, respectively. Multiple reactions monitoring (MRM) was used during the mass spectrometric analysis of the target compounds. The selected MRM transitions, including their associated parameters, are summarized in Table 6, Appendix A.

3. Results

3.1 Method Characteristics

3.1.1 Extraction Recovery Data

Extraction recovery of PBDEs from brain tissue was studied by comparing the concentration measured in the final extracts to the known concentration of the standard with which the samples were spiked. Samples were spiked at two different concentration levels, low and high and were spiked at different times throughout the extraction to see if the recoveries differed in concentration as a function of time. Results indicated that more than 80% of PBDEs tested could be extracted from tissue using this current method. Figure 2 shows the percent recovery of each congener as histograms at both the low and high spiking concentrations. The RSDs of the extraction were <10%, indicating good repeatability of extractions.

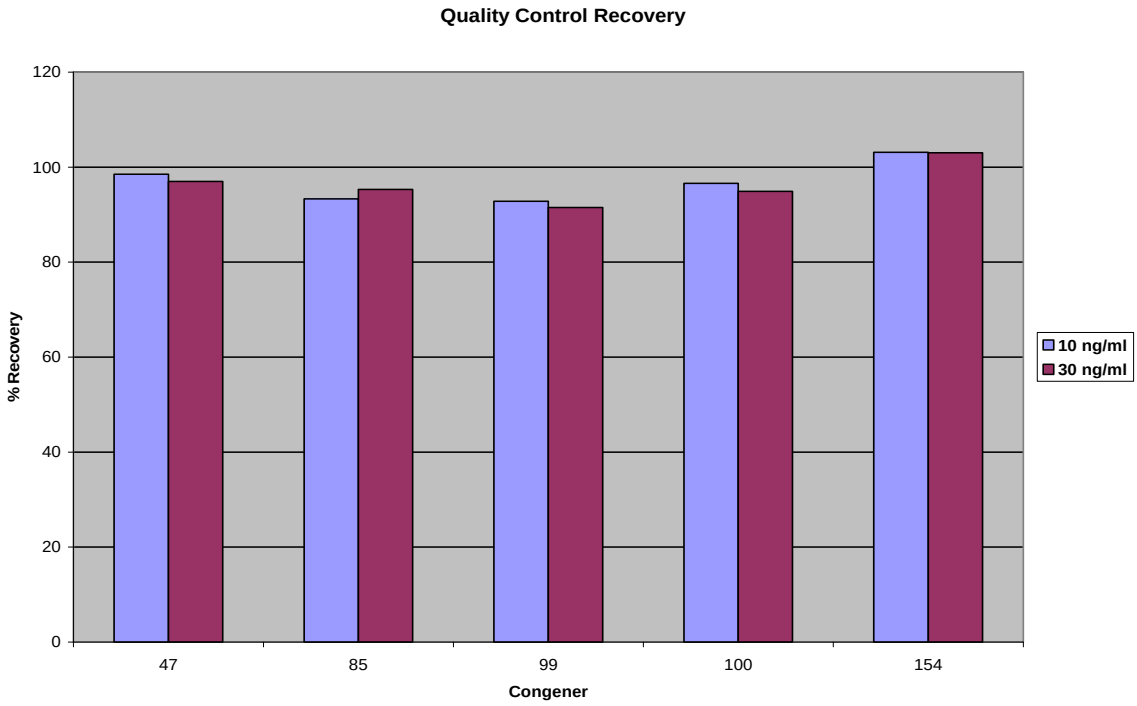

Figure 2. The recovery of each PBDE congener that was spiked into rat brain is shown. Approximately quantitative recovery was calculated for each congener at two concentration levels indicating that our extraction procedure was highly efficient. The relative standard deviation of the recovery at each concentration level for each congener was <10%.

Table 1 shows the LODs and LOQs for each congener as well as the average signal-to-noise ratio (S/N) produced by the lowest standard giving a S/N> 3 from which the LOD was extrapolated.

BDE	LOQ (ng) (on-column)	Mean S/N	Calc. LOD (pg) (on-column)	Calc. LOD (ng) (on-column)	LOD (ng/g brain)	LOQ (ng/g brain)
47	0.002	7.200	0.83	0.00083	0.83	2
99	0.004	11.804	1.02	0.00102	1.02	4
100	0.004	13.694	0.88	0.00088	0.88	4
85	0.020	27.180	2.21	0.00221	2.21	20
154	0.020	23.264	2.58	0.00258	2.58	20

Table 1 – Limits of detection and quantification for each congener. The second column shows the LOQ on-column and is not extrapolated. The Calc LOD (in columns 4 and 5) is the extrapolated value LOD. The LOD and LOQ are also expressed as ng/g of brain tissue.

3.1.2 Precision & Accuracy

The precision and accuracy of the quality control (QC) experiments are shown in Table 3 and Figure 3 and values are reported to show the day-to-day variation among the runs, the variation within the runs themselves, as well as the total variation among QC samples. The total variation as well as the inter- and intra-day relative standard deviations (RSDs) can be seen in Table 2 and Figure 3. The mean concentrations of the samples according to spiking concentration can be seen in Table 3. The best indicator of precision for the QC samples is the %RSD. The RSD is widely used in analytical chemistry to express the precision and repeatability of an assay. Low percentages indicate good precision and prove this method to be very consistent. Mean concentration values are given for samples that were spiked with the QC standards at both low (1 ng/sample) and high (3 ng/sample) concentrations. Minimal deviation from these concentrations indicates good accuracy.

		BDE	47	85	99	100	154
within	Low N=3/day	% RSD	2.34	11.85	9.13	4.33	18.30
	High N=3/day	% RSD	2.77	5.99	4.94	4.17	5.46
among	Low N=5 days	% RSD	3.99	12.00	14.87	5.17	9.30
	High N=5 days	% RSD	6.62	12.72	10.33	7.10	6.69
total	Low N=15	% RSD	4.33	15.28	16.08	6.39	15.83
	High N=15	% RSD	6.65	12.91	10.53	7.59	8.21

Table 2. Values from a five day precision study to prove the accuracy and reliability of the extraction method. Mean concentrations are given for samples that were spiked at both a low (1 ng/sample) and high (3 ng/sample) concentration along with the standard deviations and %RSD, which indicate high precision and accuracy.

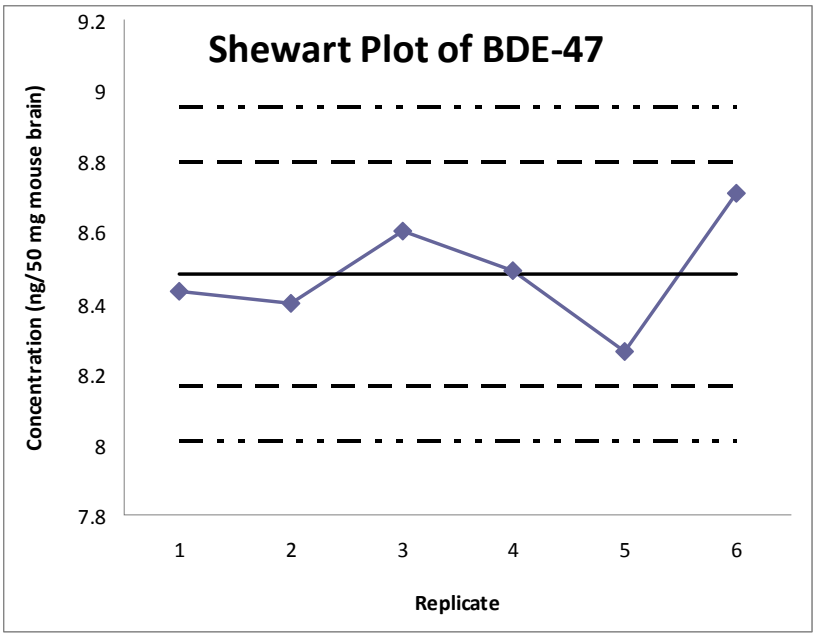

Figure 3. A clinical laboratory Shewart plot demonstrating day-to-day fluctuations in quality control (QC) materials. The mean value of the QC pool is shown by the solid line and the upper and lower 95th and 99th confidence limits are shown by the dashed lines. As can be seen, all runs reported in this thesis are "in-control."

The good precision and accuracy, along with high recoveries validate our method. These studies were done to assess any potential for error in the analysis and to quantifiably characterize system performance. One additional study was done to show the recoveries at three concentration levels, un-spiked, low and high. The results of this study showed good recovery values as predicted by their spiking concentrations, indicating that the analysis was done with high performance and low error. Samples spiked at a low concentration (1 ng/sample) as well as high (3 ng/sample) all showed good recovery with a low coefficient of variation, indicating good analysis technique. The unspiked mouse brain samples had measureable levels of PBDEs 47, 99 and 100 indicating some exposure to these compounds within the laboratory setting prior to their sacrificing. Laboratory blanks were truly without measureable analyte levels indicating no laboratory contamination of sample.

QC Level	Congener	Mean (ng)	Std Dev	RSD (%)	LoQ (ng)
Low (unspiked)	**47**	0.07	0.05	72.48	0.002
	85	<LOD	NA	NA	0.020
	99	0.07	0.04	60.00	0.004
	100	0.02	0.01	35.89	0.004
	154	<LOD	NA	NA	0.020
Low (spiked)	**47**	8.48	0.16	1.86	0.002
	85	7.83	0.61	7.83	0.020
	99	8.19	0.26	3.22	0.004
	100	8.08	0.16	1.93	0.004
	154	8.07	0.38	4.69	0.020
High (spiked)	**47**	25.67	0.64	2.49	0.002
	85	23.70	1.90	8.02	0.020
	99	24.57	1.06	4.31	0.004
	100	24.34	0.94	3.86	0.004
	154	24.62	1.32	5.36	0.020

Table 3. Validation study demonstrating the mean concentration values of samples spiked with native standard at low and high levels, 10 ng and 30 ng, respectively, as well as un-spiked samples to show background concentrations of all five congeners of interest.

3.2 Method Application

3.2.1 Demographics

The demographic characteristics of the study population are described in Table 4. The sample size was 44 and all of the brain tissue samples were taken post-mortem from the *substantia nigra* region of the brain. Decedent tissues were acquired from the Emory University Brain Bank and included 14 control samples in which no neurodegenerative disease diagnosis was given, 15 samples from patients diagnosed with Alzheimer's disease (AD) and 15 samples from patients diagnosed with Parkinson's disease (PD). Overall, the samples were predominately from white males, with an average age of 67 years.

Disease Status	Age ± Std Dev	Sex (%Male)	Race (%White)
Total	66.70 (6.21)	66	86
Control	65.14 (6.60)	50	60
Parkinson's	70.27 (5.18)	87	100
Alzheimer's	64.60 (5.51)	60	93

Table 4. Demographics, presented as mean age in years as well as sex/race expressed as a percentage of a reference group (i.e., males and whites).

3.2.2 Unknown Sample Analysis

Table 5 shows the quantitative statistics of the unknown samples arranged by disease status as well as all groups combined. Concentrations found in these samples ranged from 0.03 ng/g to 9.60 ng/g. The average summed concentrations of the congeners of interest varied in concentration with a large standard deviation, which is common among studies of this size. Most importantly, however, was the high frequency of detection, a total of 79%, indicating the prevalence of PBDE deposition in brain tissue.

Histograms showing the distribution of the data for each congener are shown in appendix A. The frequency of detection of each congener was high and most samples fell within a small range of values. These charts show the distribution of each congener and are normally distributed for BDE-47 and 99 based on a one-sample Kolmogorov-Smirnov test. Detection of BDE-100 was lower, and concentrations often approached the LOD, skewing the data. The results of the test for normality are shown below in Figure 4.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of C47 is normal with mean 1.798 and standard deviation 1.771.	One-Sample Kolmogorov-Smirnov Test	.086	Retain the null hypothesis.
2	The distribution of C99 is normal with mean 1.017 and standard deviation 0.831.	One-Sample Kolmogorov-Smirnov Test	.444	Retain the null hypothesis.
3	The distribution of C100 is normal with mean 0.285 and standard deviation 0.398.	One-Sample Kolmogorov-Smirnov Test	.013	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure 4. Test for normality of the confirmation ions of BDEs 47, 99, and 100 showing the mean and standard deviation along with significance.

Disease Status	BDE	Mean	Std Dev	Median	GM	SE	Range		Percentile		FOD %
							Min	Max	5th	95th	
All	**47**	1.80	1.77	1.16	0.73	10.35	0.001	9.60	0.001	3.66	89
	99	1.02	0.83	0.80	0.49	7.07	0.003	4.30	0.003	2.39	89
	100	0.29	0.40	0.20	0.05	11.99	0.003	2.02	0.003	1.04	59
	SUM	**6.01**	**5.43**	**4.12**	**0.15**	**180.95**	**0.001**	**9.60**	**1.26**	**16.32**	**79**
Control	**47**	2.45	2.37	1.51	1.20	7.86	0.001	9.60	0.60	5.77	93
	99	1.27	1.12	0.76	0.69	5.61	0.003	4.30	0.30	3.09	93
	100	0.41	0.56	0.25	0.07	13.76	0.003	2.02	0.003	1.42	64
	SUM	**8.00**	**7.33**	**4.66**	**0.40**	**302.72**	**0.001**	**9.60**	**1.89**	**20.84**	**83**
AD	**47**	1.37	0.69	1.12	0.80	6.40	0.001	2.75	0.59	2.55	93
	99	0.88	0.62	0.76	0.36	8.26	0.003	2.17	0.003	1.78	87
	100	0.15	0.20	0.003	0.03	10.96	0.003	0.62	0.003	0.52	47
	SUM	**4.62**	**2.28**	**3.96**	**0.09**	**47.65**	**0.001**	**2.75**	**2.43**	**8.99**	**76**
PD	**47**	1.62	1.81	0.94	0.34	21.08	0.001	7.01	0.001	4.46	80
	99	0.93	0.68	0.94	0.39	8.71	0.003	2.47	0.003	2.00	87
	100	0.31	0.34	0.26	0.07	12.01	0.003	1.27	0.003	0.89	67
	SUM	**5.52**	**5.39**	**3.07**	**0.06**	**383.47**	**0.001**	**7.01**	**0.93**	**14.29**	**78**

Table 5. Distribution percentiles of PBDE congeners and summed PBDEs in human brain tissue, stratified by disease status. Individual congeners concentrations are in ng/g brain tissue units whereas summed congener concentrations are in μmol/g brain tissue units.

PD = Parkinson's Disease; AD = Alzheimer's Disease; FOD = frequency of detection; SUM = the molar sums of congeners 47, 99 and 100.

4. Discussion

4.1 Method

This thesis provides the first evidence of our ability to extract PBDEs from human brain tissue. Previously, no method existed for this purpose and this development of a method based only on the literature surrounding PBDEs in other biomatrices is quite unique. There were many difficulties experienced along the way and caused the process to be a long and arduous one with a lot of trial and error. At least fifteen separate extractions were done before finalizing the method. During this time, we tested for matrix effects, effects of evaporation and solvent exchange, reconstitution solvent efficacy, temperature effects, effects of filtering under different environmental conditions and effects resulting from the duration of the procedure. Over half a dozen practice extractions were performed in addition to recovery studies, accuracy and precision studies, and validation studies.

The first version of the method was very crude and was an outline of the procedure that would require many changes and alterations during the development of the final method. The initial method called for 200 mg of brain tissue which was changed to 50 mg for the final method. The spiking volume of the standards was changed from 50 µl to 100 µl. The volume of solvent used in the liquid extraction phase was changed from 20 mL to 12 mL. And the method of reconstitution was changed multiple times. All of the changes made were to address one specific problem and that was the amount of fat in the sample. Since brain is such a lipid-rich matrix, it is difficult to extract non-polar compounds such as PBDEs without extracting the fat at the same time. At first, the samples would retain high quantities of fat which would make the samples dirty and difficult to analyze. A reduction in the amount of matrix along with physical controls such filtering helped to reduce the amount of fat in the final extract, making it easier to analyze on the GC-MS/MS. However, with a loss of fat came a loss of some of the PBDE congeners. It was difficult to detect the more highly brominated congeners (153, 154) on the analysis of these

low-fat samples because they would be retained in the lipids and the equipment had to be adjusted to accommodate and make up for this loss in concentration. Thus, the process of developing the method was arduous and very time-consuming. It took roughly 6 months to obtain the final method that was used to analyze the human tissue samples.

Some of the most important changes made throughout this process included the amounts of solvents used, the physical treatment of samples and the length of time these treatments required, the temperature and pressure profile of each extraction phase, and the development of standard operating procedures for all laboratory equipment used during the extraction, such as centrifuges, vortex mixers, evaporators, sonicators, etc. The proper spiking concentrations of standards had to be modified to find the volume that gave the best results during analysis, so there was also a large deal of technical adjustment of equipment in addition to mechanical and physical alteration of the method. Once the method was finalized, we were able to prove its effectiveness through quantifying results.

As previously mentioned, accuracy, precision, and validation studies confirmed the efficiency of the method. Accuracy was consistently above 80% across the board. This is within the range (100 ± 20%) deemed acceptable by the FDA in pharmacologic studies. Biases in accuracy may exist, as measurements are highly matrix dependent. To develop the method, we mimicked human brain tissue by using rodent brain tissue, a similar matrix, but not 100% identical. To attain ~100% accuracy would require "standard addition" (essentially using each brain as its own calibrant and measuring it >5 times after subsequent spiking) which is cumbersome, time consuming and cost prohibitive (from both an analytic measurement and brain usage standpoint). Matrix effects are very important to consider in a study such as this, when no previous studies have been done to assess these effects. Because human brain tissue is not readily available for use, we were required to use other mammalian brain tissue as a basis for our analyses. The variation among these tissue types contributes to issues in both the extraction procedure as well as the analytical procedure.

The complications encountered when using lipid-rich matrices were overcome by careful, selective extraction procedure/cleanup in addition to the use of MS/MS analytical techniques. This is because lipids interfere with mass calibration and instrument "lock" in more commonly used high resolution mass spectrometers (magnetic sectors). MS/MS, while expensive equipment, is less expensive than high resolution MS (~300K vs 600K), is more rugged, and less sensitive to atmospheric changes in the laboratory (e.g., temperature, humidity, vibration).

4.2 Data

The aim of this thesis was to develop a method for extracting PBDEs from human brain tissue and to prove that the method works by quantifying the exposure in unknown samples. As the data show, we were able to successfully quantify PBDE levels in the brain with good accuracy and precision. We were able to detect PBDEs in most of the samples tested, and for most of the congeners of interest. While variation in the data does exist, the fact that we now have data to study denotes a successful method development. It also provides implications for the ability of BFRs to cross the blood-brain barrier, which had up until now only been a source of speculation. The distribution of the data was shown to be relatively normal for congeners 47 and 99, and somewhat skewed for BDE-100, but as PBDEs continue to be studied, the analytical methods for detecting biologically relevant congeners will also improve and be able to quantify even lower concentrations than we are currently able to. Just based upon the data attained through this study, no assumptions can be made regarding the association between exposure and disease, although the data showed that the control group had a higher average concentration than the diseased groups, the opposite of what we hypothesized. Further analysis with lipid adjusted data must be done in order to fully explore this hypothesis.

4.3 Limitations

This thesis aimed to develop a method for detecting PBDEs in human brain tissue and the method developed for this was confirmed a success by the quantification of PBDEs in the samples analyzed. Regarding this aspect, there are limitations to what we were able to achieve. We decided to focus on six biologically relevant congeners of PBDEs, as demonstrated by other studies done on human plasma. The six congeners were 47, 85, 99, 100, 153, and 154. Using this novel method, we were able to successfully quantify BDEs 47, 99, and 100. The remaining congeners were not able to be detected by standard analysis techniques above the limit of detection. Because of the complex chromatography associated with this matrix, we decided to focus only on those congeners that had the greatest frequency of detection. Another limitation was the use of nonhuman brain tissue for calibration. All of the tissue used to create the analysis profile was mammalian, but slight differences in composition could account for variation in the data. The unknown samples that were analyzed were not lipid-adjusted (because of time constraints as well as cost) to account for differences in lipid content, which may have biased the resulting data. This small sample size (n=44) of this study limits the ability to make claims about the association between exposure and disease; further analysis is warranted..

4.4 Future directions

Further studies will be conducted using this method and new information regarding extraction and analytical techniques will be added. One of the most important additions will be the lipid-adjustment of samples to account for variations in lipid content, allowing for the normalization of data. A larger sample size will provide more insight into the distribution of PBDEs within brain tissue. These data, when combined with an additional 75 samples from the Columbia University Brain Bank, will be used to determine if an association exists between brain concentrations of PBDEs and development of neurological disease. This thesis has served as a beginning to this study and I will continue to work on the study alongside investigators.

I am currently still working in the laboratory where this research was conducted and plan to assist in the remainder of the study. Much is known about PBDE toxicology through rat studies and when the additional samples are analyzed, we will be able to make an initial determination regarding the association between PBDE exposure and neurological disease. After additional data are obtained, one or more publications on this study will be submitted and hopefully added to what modest literature exists regarding this topic. Because we are now able to detect a compound previously unknown to cross the blood-brain barrier, we can begin to focus on other environmentally persistent compound with the ability to bioaccumulate such as endosulfan and perfluorinated compounds, which may also present in brain tissue.

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Appendix A

					Fragmentation from our Experiment (3 best transitions)													
PBDE		RT		MW	Quant			Qual_1				Qual_2						
					Q1	Q3	CE	Q1	Q3	CE		Q1	Q3	CE		Q1	Q3	CE
BDE-47		4.56		207.3	485.6	326.0	25	325.7	217.0	25					47 ISTD	497.5	338.0	25
BDE-100		5.37		284.8	563.6	404.0	20	565.5	406.0	25		403.6	137.0	55	100 ISTD	577.6	418.0	20
BDE-99		5.65		215.7	563.6	404.0	20	565.6	406.0	25		403.6	137.0	55	99 ISTD	575.5	416.0	35
BDE-85		6.25		209.2	565.6	406.0	20	563.6	404.0	40		405.6	137.0	25				
BDE-154		6.59		246.3	643.5	484.0	20	641.5	482.0	30		483.5	377.0	30				
															154/153 ISTD	655.5	496.0	20
BDE-153		7.12		223.2	643.5	484.0	20	483.5	324.0	40								

Table 6. Descriptive Mass Spectrometer Parameters

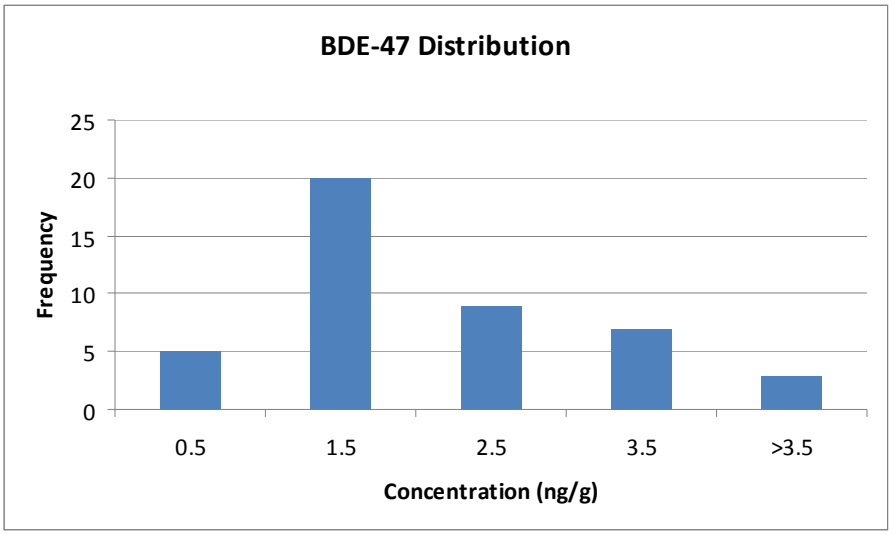


Figure 5. Distribution of BDE-47

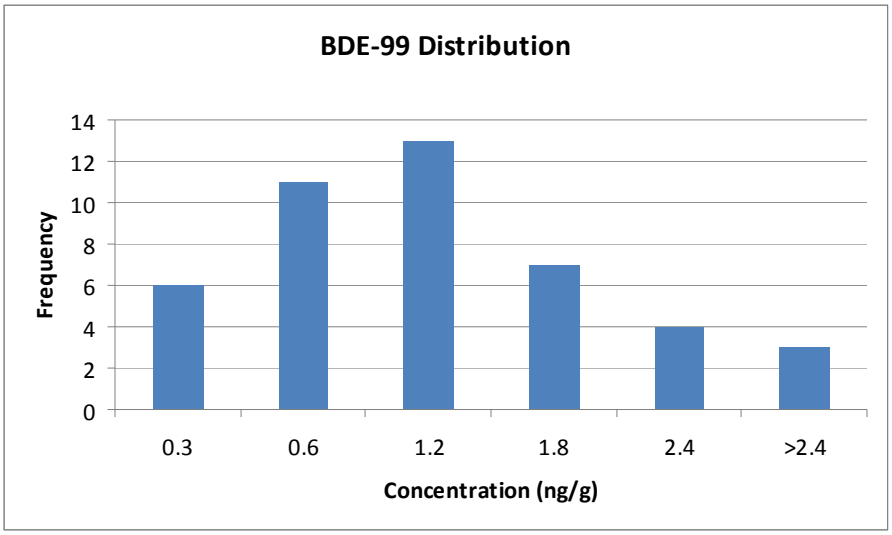


Figure 6. Distribution of BDE-99

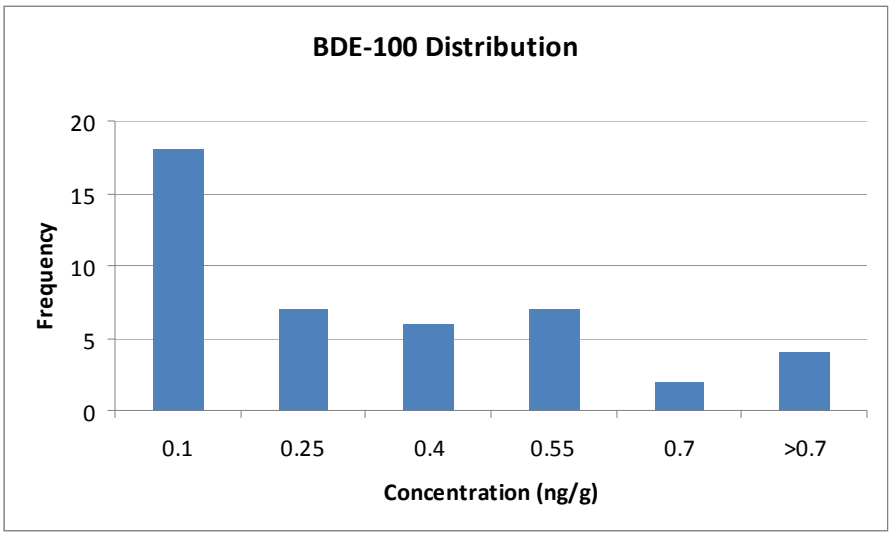


Figure 7. Distribution of BDE-100

Appendix B

Extraction Procedure of PBDEs from Brain Tissue

Materials:
Tissue Homogenizer
Sonicator
Centrifuge
Restek 3mL 500mg Florisil SPE Cartridge
3 sets of labeled test tubes in rack for: sample pretreatment, protein precipitation and SPE collection

Solvents:
Acetone
Hexane
Toluene

Procedure:

Sample Pre-treatment
Blank Sample:

- Use ~50mg pooled matrix: spike with 100ul ISTD only

Standard Curve and QCs:

- Use ~50mg pooled matrix: spike with 100ul ISTD and 100ul QC STD

Unknown Samples:

- Add ~50mg of frozen unknown brain tissue: spike with 100ul ISTD

Protein Precipitation (Stage A)

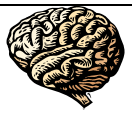
1. Add 0.5mL acetone and 0.5mL hexane to homogenization tube (16x100mm) containing brain sample
2. Homogenize sample and transfer to labeled 15ml conical vial
3. Rinse homogenization tube with additional 1.0mL acetone and 1.0mL hexane and transfer remaining tissue to conical vial
4. Spike sample with ISTD (and native STD for QCs)
5. Sonicate 15min
6. Vortex for 2 min
7. Centrifuge for 10min at 2500rpm at 4C
8. Filter supernatant through a 20um pore fritz cartridge into a clean, labeled 15ml conical glass test tube (that has been pre-weighed and recorded)

Cleanup (Stage B)

9. For the remaining tissue in the tube, repeat steps 5-7 an additional 3 times using 1.5ml of hexane and 1.5ml of acetone (total of 12ml)
10. Turbovap samples (45C) and bring to dryness.
11. Weigh test tube when completely dry, record residue weight.

Extraction (Stage C)

12. Reconstitute in 0.5mL hexane and 0.5mL acetone and vortex
13. Set up collection manifold using Restek 500mg 6cc Florisil cartridges
14. Condition cartridges using 1ml hexane
15. Insert labeled test tubes into manifold collection chamber
16. Pass reconstituted samples through Florisil column bed
17. Elute with 2mL of hexane 5 times
18. Turbovap to ~1ml (~10min @ 35C, 10psi), then add 100ul toluene
19. Turbovap down to 100ul
20. Transfer to labeled GC vials for analysis



50mg Sample

16x100mm Test Tube

0.5mL Acetone

0.5mL Hexane

Add ISTD (100µL)

Homogenize and transfer to 15mL conical tube

Rinse 16x100mm test tube with 1.0mL acetone and 1.0mL hexane; Add to 15mL tube

Sonicate (15min)

Vortex (2 min)

Centrifuge (10 min @ 2400rpm, 4°C)

Add 1.5mL of both Acetone and Hexane to remaining tissue and repeat 3x, totaling 12mL of supernatant

New set of test tubes, clean, labeled, with weight recorded

Filter supernatant through 20µm pore frit

Evaporate at 45°C, 15psi
Record weight when dry

Reconstitute with 0.5mL acetone, 0.5mL hexane and vortex briefly

Set up collection manifold with clean 15mL conical tubes and Florisil cartridges and condition with 1mL hexane

Load samples into cartridges

Elute with 2mL hexane, 5 times

Evaporate to ~1mL
(~10min @ 35C, 10psi)

Add 100 µL toluene and vortex

Transfer samples to GC vials with silanized glass inserts

Evaporate to 100 µL
(Requires constant surveillance and frequent checking)