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Dried Blood Spots as a Matrix for Biomonitoring of Polyhalogenated Biphenyls

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Abstract

Dried Blood Spots as a Matrix for Biomonitoring of Polyhalogenated Biphenyls

By Andrea Marina Elizondo

Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) are classes of persistent organic pollutants that are known endocrine disruptors and have been shown to cause neurodevelopmental toxicity and increased cancer risk. Biomonitoring of chemicals such as PCBs and PBBs has become an increasingly popular and important tool for exposure assessment. Historically, serum or plasma have been used as the primary matrices for biomonitoring of exposures, but various limitations associated with the use of these matrices have led to the research and development of simpler and more cost effective tools for exposure assessment. Dried blood spots (DBS), drops of whole blood collected on pieces of filter paper are a simpler and more cost effective tool for the future of biomonitoring, yet little has been done in the way of method testing and development due to the extremely low blood volume of each spot (~65 μL). The objective of this study was to measure levels of PBB-153, PCB-118, PCB-138, PCB-153, and PCB-180 in DBS collected through the Michigan PBB registry using a newly developed method utilizing gas chromatography-electron impact ionization-tandem mass spectrometry (GC-MS/MS). The results of the DBS analysis were compared to participant matched serum, analyzed using the same method. Extraction recoveries using DBS ranged from 90.0%-93.7%, accuracies for all five target compounds ranged from 80.0%-110.5%, and inter-day precisions ranged from 2.4%-16.4%, all of which fall within the recommended acceptable standards. Statistical analysis showed strong positive correlations between the DBS and serum methods, and Bland-Altman analysis indicated strong agreement between methods. Our study was the first to use GC/MS-MS for the analysis of these compounds in DBS, and has provided a cost effective, selective, and robust method for biomonitoring of PCBs and PBBs.

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Introduction & Background

Biomonitoring

Exposure assessment is an increasingly important and difficult part of environmental health, often being referred to as its Achilles' heel [1]. Due to great advances in analytical tools for exposure assessment, biomonitoring has quickly become a frequently used tool for exposure assessment [2]. Biomonitoring of exposure, which is defined as the assessment of human exposure to chemicals by measuring chemicals, their metabolites, or reaction products after the bodily absorption of a chemical, is not a new field of study [3]. For example, literature dating as far back as 1920 has demonstrated the analysis of blood lead and urinary mercury levels for occupational health research [4]. Serum, plasma, and urine have long been the primary matrices used in biomonitoring of exposure, allowing investigators to relate chemical concentrations to internal dose and ultimately to the effects of the exposures [5]. In addition to the primary matrices previously mentioned, milk, saliva, adipose and other tissues can be used depending upon the chemical of interest and its pharmacokinetics [5]. With major advances being made in biomonitoring, its potential uses and applications will continuously improve exposure assessment.

Polybrominated Biphenyls

Polybrominated Biphenyls (PBBs) are a class of chemicals that were manufactured beginning in 1970. While there are 209 potential PBB congeners,

only 42 have ever been synthesized [6]. The empirical formula for PBBs is $C_{12}H_{10-n}Br_n$ with n varying from 1-10 [7]. The general structure of PBBs is illustrated in Figure 1. Little is known about the mechanism by which PBBs enter the blood stream or how PBBs are distributed in the body, but current research suggests that the most likely mechanism for absorption is through passive diffusion after ingestion exposure. Additionally, while the mechanism of toxicity has been thoroughly researched, it is still not completely understood. It is thought that PBBs act much like other structurally related halogenated aromatic hydrocarbons such as PCBs. Some studies have suggested that much like PCBs, PBB congeners bind to the aryl hydrocarbon receptor, initiating transcriptional upregulation of genes that mediate endocrine pathways, oxidative stress responses, and cell cycle regulations [6].

PBBs were made to serve as flame-retardants for consumer products such as computers, furniture and foams. Unlike most chemicals, PBBs are not chemically bound to these products, but rather mixed into plastics, meaning that they are readily shed into the environment [8]. PBBs do not have any known natural environmental source and therefore enter the environment specifically through their manufacture, use, and improper disposal. Although production of PBBs in North America no longer occurs, those living in close proximity to areas where previous contamination has occurred, particularly in the state of Michigan, remain at risk of exposure [8]. Exposure to PBBs occurs through ingestion of contaminated food, water or soil, and inhalation of contaminated air. While a partial breakdown and release of the PBBs occurs in the body, PBBs tend to be

stored in adipose tissue and breast milk and have been found to have transplacental capabilities [8]. This class of persistent chemicals with a half-life of approximately 11 years has been shown to have numerous negative health effects in animal studies, raising concerns over their human health effects.

An array of human and animal studies have found that short and long term exposure to PBBs caused human health effects such as reduced body weight, immunological and lymphoreticular effects such as increased levels of IgM, IgA and IgG, dermal effects such as acne and darkening or thickening of the skin, neurologic and behavioral symptoms due to in utero exposure, and increased risk of certain cancers such as breast and liver cancers [6, 8-10].

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are a class of persistent organic pollutants (POPs) that are considered to be among the most environmentally ubiquitous chemicals to date, with half lives on the order of decades [11]. PCBs are a complex class of chemicals, with 209 identified congeners. The empirical formula for PCBs is $C_{12}H_{10-n}Cl_n$ with n varying from 1-10 [7]. The general physical structure of PCBs is shown in Figure 2. PCBs are highly lipophilic compounds that are readily absorbed by the gastrointestinal tract and are stored in high concentrations in adipose tissue [12]. The PCB congeners undergo metabolism by cytochrome P-450 in the liver, which allows for subsequent conjugation with glutathione and glucoronic acid. Because the rate of metabolism determines the overall toxicity of each congener, the number and position pattern of the chlorine atom

substitutions on the biphenyl rings determine the overall toxicity of each PCB congener [12, 13]. While the mechanisms of toxicities for PCB congeners that show estrogenic and neurotoxic activity and carcinogenicity are not quite clear, research has shown that the group of PCB congeners considered to be “dioxin-like” (i.e., coplanar non ortho-substituted and mono-ortho-substituted PCBs) cause enhancement of gene expression due to binding of the aryl hydrocarbon receptor (AhR)[12].

PCBs were manufactured beginning in 1929 for uses in products such as transformers and capacitors, voltage regulators, cable and thermal insulation, plastics, and carbonless copy paper due to their non-flammability, chemical stability, and high boiling point [14]. Production of PCBs was banned in 1979 due to concerns over human toxicity and chemical persistence of PCBs in the environment, however, this does not mean that exposure ceased with production. Thirty-six years after production ceased, PCBs are still being released into the environment by hazardous waste sites that have not been properly maintained, illegal dumping of PCB containing wastes, and from electrical transformers that contain PCB products [14]. The primary routes of human exposure to PCBs are through ingestion of contaminated food products, particularly meat and fish, and by inhalation of contaminated air [11, 12]. Extensive human and animal studies have shows that PCBs can have various health effects such as acne-like skin conditions, liver, stomach and thyroid gland injuries, reduction in immune system function, impaired reproduction, endocrine changes, certain cancers and developmental neurotoxicity [12].

Biomarker Research

In the past, studies conducted by social and behavioral scientists have largely focused on data provided through clinical records and self-reported information, however, the increased use of biomarkers in recent decades presents a method for integrating biological data into population-based health research [15]. Additionally, the use of biomarkers has provided a way to tap into physiological processes and health effects that may reveal the way that exposures determine a persons individual risks or future health outcomes. Between 1986 and 2009, there were approximately 28,856 grants awarded by NIH for biomarker related research, indicating the growing interest and importance in the development of these analytical methods [16].

PBB Contamination Incident of the Early 1970s

From 1936 to 1978, the Michigan Chemical Corporation, later known as Vesicol Chemical Corporation, manufactured a magnesium oxide nutrition supplement for livestock feed named NutriMaster® and a brominated flame retardant containing PBBs called FireMaster® within the same manufacturing warehouse. In the early 1970s, approximately 1,000 pounds of FireMaster® was mistakenly sent to the Michigan Farm Bureau where it was subsequently mixed into animal feed that was then shipped to feed mills across the state. Within days of ingestion of the contaminated feed, cattle began to show clinical signs such as decreased milk production, early embryonic absorption, weight loss, increased pregnancy complications and alterations in metabolic excretions [17, 18]. This incident

resulted in the contamination of approximately 40,000 cattle, 2,000,000 chickens and thousands of other farm animals, which had to be quarantined and destroyed [19]. It is estimated that by the end of 1974, approximately 6.5 million Michigan residents had consumed PBB contaminated food products such as beef, poultry, and dairy [18]. In response to this incident, the Michigan Department of Community Health created a PBB registry that included persons who either lived on or received food from quarantined farms, as well as chemical workers and their children. Each recruited participant completed a questionnaire that included demographic information and a complete medical history. In addition to the questionnaire, 85% of participants provided a blood sample that was analyzed for PBBs using gas chromatography with electron capture detection [20]. After more than 30 years, the management of the PBB registry was transferred to Emory University due to a lack of funding and resources. In order to remain in the PBB registry, all participants were re-contacted for consent. To date, approximately 40% of the PBB registry members have re-consented, and their records are now used by Emory University for important studies in public health [20].

Current Study

Dried blood spots (DBS) are drops of whole blood that are collected on pieces of filter paper (Guthrie cards) from a simple finger or heel prick. For decades, DBS have been collected as part of state new born screening programs in the United States that test for metabolic disorders such as phenylketonuria and tyrosinemia [21, 22]. After the DBS have been used by the state for metabolic analysis, the

blood spots are kept either short term (<3 years) for standard program uses, or long term (>18 years) for standard program uses and possible novel public health research [23].

In 2009, the Secretary of Health and Human Services in conjunction with the newly formed Advisory Committee on Heritable Disorders in New Born Children developed and released recommendations on retention policies and future uses of DBS. The recommendations focused on confidentiality and genetic testing, but did not make recommendations on the potential use of DBS for biomonitoring [23]. As a result of public concerns over the lack of parental knowledge of the retention and use of DBS for public health research, the Newborn Screening Saves Lives Act (NSSLA) went into effect in March of 2015. The NSSLA states that newborn dried blood spots are now always considered human subjects and states that IRB's can no longer waive informed consent in research that involves newborn dried blood spots [24]. While serum or plasma have long been the primary matrix used in biomonitoring, current research suggests that DBS could present a more cost effective and less invasive option for biomonitoring and data collection. Advantages to using DBS include ease of collection by both medical and non-medical personnel, which may promote greater enrollment of infants and children in studies, the cost efficiency of DBS as compared to blood collection supplies, availability of DBS from state new born screening programs, and ease of shipping [20]. Additionally, unlike the analysis of serum or plasma, which only provides information on current body burden and leaves timing of exposure unknown, DBS have the potential capability of being used for studies on

multigenerational health effects as a result of developmental exposures to endocrine disrupting chemicals which have been shown to cause phenotypes that persist up to five generations in animal studies [20, 25, 26]. While DBS are advantageous in many aspects, few have attempted to use them for biomonitoring because each blood spot provides about 100 μL of blood (~45 μL of serum) which is approximately 1/25th of the amount of blood normally used for analysis of POPs [20].

The current study focuses on the analysis of DBS as an effective tool that could dramatically reduce sample collection and shipment costs, increase the number of participants in studies and provide an unobtrusive mechanism for evaluating prenatal exposure to PBBs based on the Michigan PBB registry and PCBs, chemicals in which all people are considered to be exposed. It would also be the first fully characterized method for analysis of polyhalogenated biphenyls in DBS. It is important to note that in order to qualify as an effective tool for exposure assessment, values quantified from DBS must be at least equivalent to those measured in the gold standard, serum.

Public Health Relevance

Exposure to POPs is inevitable, yet much is unknown about the long-term health effects of these exposures. It is important to study multigenerational effects of the chemicals all around us. Development of less invasive and less expensive technologies will promote great advancements for exposure assessment. For the first time, researchers could have direct measures of prenatal exposures to PBBs

and PCBs that are not directly dependent on maternal levels and estimated half lives, providing an unobtrusive mechanism for evaluating prenatal environmental exposures in newborns, and a method for follow up of individuals who were excessively and overtly exposed to chemicals during their lifetimes using archived blood spots. In addition, this study would afford several advances in the understanding of the PBB exposures in Michigan.

Study Design & Methods

The methods utilized in the current study for the quantification of PBBs and PCBs in human matrices were developed by the Laboratory of Exposure Assessment and Development in Environmental Research (LEADER) at Emory University's Rollins School of Public Health.

Study Population

Fifty-three participants who were recruited into the PBB registry were approached about participating in this study, with the targeted enrollment representing the demographics of Michigan based on the 2010 US Census. All participants gave informed consent to access of their information in their PBB registry record.

Sample Collection and Shipment

Each participant provided a 10 mL blood draw using a serum-separator tube with a butterfly attachment. The blood was allowed to clot for 30 minutes and was

subsequently centrifuged, resulting in approximately 4 mL of serum per participant. Participant's blood samples were shipped overnight via FedEx over dry ice and kept at -20°C until analysis. An additional 2 mL blood draw was taken from each participant in order to create the dried blood spots for future analysis. Using the 2 mL whole blood sample, six spots on each of two Guthrie cards were completely filled (~65 µL of whole blood per blood spot) using a pipette, and were allowed to dry. Once dried, one card from each participant was shipped to Emory University via USPS. All DBS cards were subsequently stored at room temperature in the laboratory analysis.

Chemicals Used in Present Study

A Milli-Q Ultrapure water purification system (Millipore, Billerica, MA) was used to generate water. Analytical grade dichloromethane, hexane and isooctane were purchased from Aldrich, Inc (St. Louis, MO). Sulfuric acid and formic acid were purchased from Fisher Scientific (Lawn, NJ). Bondesil silica sorbent was purchased from Agilent (Santa Cruz, CA). Isolute ® 200 mg silica cartridges were purchased from Biotage (Charlotte, NC). Anhydrous sodium sulfate was obtained from Sigma-Aldrich. Whatman 903 protein saver cards were obtained from VWR International (Radnor, PA). In preparation for the blood spot matrix quality controls, whole blood was drawn in-house. Pooled human sera was provided by the Red Cross (Interstate Blood Bank LLC, Memphis, TN). The standard reference material (SRM 1958, organic contaminants in fortified human sera) was obtained from the National Institute of Standards and Technology

(NIST, Gaithersburg, MD). Nitrogen and helium gas were purchased from NexAir, Inc. (Suwanee, GA) and were 99.999% ultra-high purity.

All PBB and PCB native standards were ≥ 95% pure. PBB congener 153 and PCB congeners 118, 138, 153 and 180 were purchased from Cambridge Isotope Laboratories (Andover, MA) as individual congeners at 100 µg/mL in isooctane. In addition, an individual PBB 153 ^{13}C-ring labeled standard at 40 µg/mL in nonane (99% purity) and a mixed PCB congener ^{13}C-ring labeled standard at 5 µg/mL in nonane (98% purity) were also purchased from Cambridge Isotope Laboratories.

Preparation of Standards Solution and Quality Control Measures

Using serial dilution of a 0.5 ng/µL stock solution in acetonitrile: dichloromethane (4:1), native PBB-PCB mixed calibration standards were prepared. In order to simplify our overall quantification scheme, solvent-based standards were used for all calibrants with each concentration expressed in serum-equivalents. These were prepared separately for serum and DBS analyses. The highest calibrant concentration was equivalent to that in the final extract from a serum sample whose concentration was 12.5 ng/mL. Serum-equivalent calibration standard concentrations ranged from 0.005 ng/mL-12.5 ng/mL across 11 points for serum and ranged from 0.005 ng/mL-5 ng/mL across 10 points for DBS. A labeled standard spiking solution with a concentration of 0.2 ng/mL in acetonitrile: dichloromethane (4:1) was prepared.

Four quality control (QC) spiking solutions containing the native PBB-PCB mixture were prepared with standard spiking solutions by serial dilution of the initial stock solution. The DBS used for QC were created using whole blood drawn from a single individual and were uniformly 65 μL. Two were used with the serum matrix and the remaining two were used with the DBS matrix. The nominal concentrations of the matrix-based QC samples when spiked into serum matrix were 0.5 ng/mL and 1.0 ng/mL when spiked into serum matrix, and 0.05 ng/mL and 0.01 ng/mL when spiked into blood spot matrix. The standard stock solution and spiking solutions were dispensed into amber vials and stored at 4°C until used.

PBB and PCB Extraction from Serum and DBS

In order to remove the blood from the spot, the entire spot was cut from the Guthrie card using sterilized scissors, was quartered, and placed in a test tube where it was then dissolved using phosphate buffered saline at physiologic pH (7.4). Additionally, a 1.0 mL serum sample from each participant was analyzed by the following methods. Each DBS sample was spiked with 25 μL labeled standard solution, which resulted in a 5 ng/mL internal standard concentration. Unknown serum samples were spiked with 50 μL of labeled standard solution, resulting in 10 ng/mL internal standard concentration. The samples were briefly vortex mixed, then 2 mL of formic acid and 2mL of a formic acid: water solution (50:50) were added to the blood spot samples and serum samples, respectively. The samples were then vortex mixed again and 5mL of hexane was added to each

sample. The DBS samples were then sonicated for 5 minutes, while the serum samples were sonicated for 1 minute. Following sonication, the samples were vortex mixed at 2,000 rpm for 10 minutes using a multi-vortexer (Benchmark BenchMixer, Edison, NJ). Samples were then centrifuged, and the organic layer was transferred to a clean test tube using a Pasteur pipette. The addition of 5 mL of hexane to each sample, sonication, vortex mixing and centrifugation was repeated as before, and the organic layer was pipetted off and was added to the first sample.

Solid-phase extraction was performed using in-house prepared acidified-silica/silica columns. The acidified silica (silica/sulfuric acid 2:1 by weight) was prepared by adding concentrated sulfuric acid to Bondesil silica and heating it at 100°C overnight. Isolute cartidges (10 mL) were packed with 1.8 g of the acidified silica and topped with 0.5 g of anhydrous sodium sulfate. These cartridges were then conditioned using 5 mL of hexane immediately prior to sample cleanup. The samples were loaded into the cartridges and sample breakthrough was collected. Using 1mL of hexane, sample tubes were rinsed, briefly vortex mixed and shaken and loaded into the cartridge. After the samples loaded, the breakthrough collection tubes were inserted into the TurboVap® (Zymark, Framingham, MA), set to 30°C and 15 psi to begin evaporation. Elution of the cartridges with 10 mL of a 1:19 dichloromethane:hexane solution followed, with the eluate being collected and combined with the breakthrough already evaporating until it was brought to total dryness in the TurboVap®. For instrumental analysis, DBS

samples were reconstituted to 20 μL of isooctane, and serum samples were reconstituted to 50 μL of isooctane.

GC-MS/MS Analysis

An Agilent 7890A gas chromatograph (GC) coupled to an Agilent 7000B tandem mass spectrometer (MS/MS) (Agilent Technologies, Santa Clara, CA) programmed and controlled using MassHunter Workstation Software version B.05.00 was used for GC-MS/MS analysis. The calibration and tune of the instrument was performed in EI with the High Sensitivity Autotune mode. Additionally, instrument performance was checked prior to each analysis.

In order to optimize separation, a polyimide-coated fused silica phase analytical column (5%-phenyl-dimethylpolysiloxane, 15m x 0.250 ID x 0.10 μm film thickness, ZB-5HT Inferno, Phenomenex, Torrance, CA) was used on the GC system. A 2 μL injection with an injection port temperature set to 325°C under pulsed splitless mode was used. The helium carrier gas flow rate was 2.25 mL/min with a quench nitrogen gas flow rate of 1.5 mL/min. The source temperature was set to 230°C and the quadrupole temperature was set to 150°C. The oven temperature scheme was as follows: 90°C (0.1 min), ramped to 340°C (20°C/min) and held for 5 min. In total, the run time was 17.6 min.

The MS/MS quantification method was created by using individual injections of each target compound in full scan mode. This allowed for the obtention of

retention times and allowed for the selection of the optimal precursor ions, generally selecting the most intense ion with the highest *m/z*. Product ion scans were performed using different collision energies set to determine the most selective product ions. Ions were selected based on their intensity, peak shape, and signal-to-noise ratio. Two transitions were then selected for each native analyte for quantification and confirmation. Only one transition was selected for each labeled analyte (^{13}C-PBB-153, ^{13}C-PCB-118, ^{13}C-PCB-138, ^{13}C-PCB 153, and ^{13}C-PCB 180). All transitions were monitored in multi-segment analysis using multiple segment analysis using multiple reaction monitoring (MRM) mode. The transitions are shown in Table 1.

Data Processing

MassHunter Workstation Software – Quantitative Analysis version B.05.00 was used for data processing. Retention time, signal-to-noise ratio, relative retention value, peak algorithm, quantitation transition and confirmation transition were generally used for characterization of each compound.

Method Validation and Data Analysis

All statistical analyses were completed using Microsoft Excel 2011 (Version 14.4.9) and SAS Statistical Software (Version 9.4). The limit of detection (LOD), accuracy, and precision were determined for both the DBS and serum samples. Spearman correlations and Bland-Altman tests were used in order to determine statistical associations and agreement between the different methods for all five analytes. In order to run statistical analysis, the DBS concentrations were

corrected to equal approximately 1mL of serum. Each DBS was created using 65 μL of whole blood. On average, serum makes up approximately 40% or 26μL of each 65 μL blood spot, therefore leaving a correction factor of 38.5. Additionally, permissive data (data with a signal-to-noise ratio less than 3) were used for all analysis. The determination for use of permissive data was due to the fact that the low volume of blood led to background noise so low that it would not allow a signal-to-noise ratio (S/N ratio) of 3 or greater. Despite the low S/N ratio, clear peaks were visible in chromatogram analysis.

Extraction Recovery:

The DBS extraction recovery of this method was determined at the spiked concentration of 0.05 ng/mL. Five DBS samples were spiked with mixtures of native and labeled compounds, and extracted according to this method. Five DBS samples that were spiked with only internal standard were concurrently analyzed deviating from the method only in that prior to the evaporation, the extracts were spiked with the same mixture of native standards mentioned earlier. These samples represented an extraction recovery of 100%. Immediately after evaporation, all samples were reconstituted and analyzed. Extraction recovery was calculated by comparing the responses of the samples spiked before extraction to the responses of the samples spiked after the extraction.

Limit of Detection:

Traditionally, LODs are determined by use of a signal-to-noise ratio (S/N) of 3. Due to the low volume of blood on each DBS, the noise in the chromatogram is

essentially non-existent, in which case the instrument may consider a nearby peak as noise or may be unable to calculate noise. This often resulted in visible peaks with low or unquantifiable S/N ratios. This being considered, we developed a more subjective way of evaluating peak integrity by determining if the peak was easily discernable, had at least 10 scan points defining the peak, and had a measurable concentration in a corresponding standard solution. Thus, the LODs presented in Table 2 would more accurately be described as a minimal detectable level (MDL) due to the fact that its designation is not based upon an objective analytical process. The MDLs take into account the DBS reconstitution factor of 20 pg/mL.

Accuracy:

For all analytes, the method accuracy was determined by calculating the difference in the mean of repeat measurements of DBS (n=5) spiked with mixtures of native compounds at two concentrations (0.01 ng/mL and 0.05 ng/mL) from the expected concentration, as well as the percentage of agreement between the mean quantified values of the NIST certified reference serum (n=4) generated from our method and their specified mean values for each lot of the reference materials.

Precision:

Method precision was determined using the relative standard deviation (RSD) of repeat measurements of DBS samples spiked with QC materials at two different concentrations (0.01 ng/mL and 0.05 ng/mL). Inter-day precision was

determined using QC materials prepared and analyzed during the discontinuous sample analysis period over two months (n=5 for each concentration).

Results

Extraction Recovery of DBS

The extraction recoveries of the DBS are shown in Table 2. Table 3 presents the serum extraction recoveries for reference. Four of the five target compounds (PBB-153, PCB-138, PCB-153 and PCB-180) were found to have good recovery from the DBS using the PBB-PCB extraction method. All calculated recoveries ranged from 90%-93.7%. Due to instrumental issues, one target compound (PCB-118) was not recoverable in the post-experiment extraction recovery. Although PCB-118 was not recoverable, the inter-day accuracy and precision was able to be determined using the QC spiked DBS samples at two concentrations.

Chromatographic Separation

Regarding the selectivity of the method, unfortified in-house DBS and Red Cross pooled serum samples, which served as method "blank" samples, showed no isobaric or chromatographic interferences with the target compounds. Figures 3-5 show the extracted ion chromatograms representing the target native compounds from analysis of a 0.05 ng/mL calibrant, a typical 0.05 ng/mL fortified in-house DBS sample, and an unknown DBS with the paired unknown serum

Minimum Detectible Limit

The calculated MDLs for the DBS samples using this method ranged from 14 pg/mL to 40 pg/mL (Table 2).

Accuracy and Precision

The validation results using DBS at two concentrations (0.01 ng/mL and 0.05 ng/mL) are shown in Table 2. Analyses of these DBS samples occurred in different batches across six months. For all five of the target compounds, accuracies ranged from 80.0% to 110.5%. Inter-day precision, expressed as the percent relative standard deviation, ranged from 2.4% to 16.4% across target compounds. Overall, method accuracy and precision for all target compounds meet the United States Food and Drug Administration's (FDA) guidance recommendation criteria for method accuracy (80%-120%) and method precision (<20%) in bioanalytical method development [27]. Additionally, the reported accuracies for the NIST certified reference serum fell between 80%-106% meeting the NIST guidelines.

Statistical Analysis

As previously mentioned, statistical analyses were performed using permissive data that have been adjusted to equal 1mL of serum.

Correlation Analysis:

Based on analysis of the histograms, skewness, and kurtosis of each of the analytes, it was determined that the data did not meet the assumption of

normality necessary to use Pearson's correlation. Spearman's correlation, a non-parametric method that does not require normally distributed data was used in place of Pearson's correlation. Table 4 presents the sample sizes and corresponding correlation coefficients (r) for each analyte. The Spearman correlation coefficients were statistically significant for each analyte, confirming a strong positive correlation between the use of serum analysis and DBS analysis for the detection of the polyhalogenated biphenyls in question.

Bland-Altman Analysis:

Bland Altman analysis is a method commonly used to assess the agreement or comparability between two methods. Bland Altman plots evaluating the mean differences and agreement intervals within which 95% of the differences of the analyte concentrations for each method fall are presented in Figures 6-10. The calculated upper and lower limits of agreement are presented with dashed lines, while solid lines represents the zero bias lines. Agreement between the two methods can be said to be acceptable, with no consistent bias of one approach versus the other. Four of the analytes (PBB-153, PCB-118, PCB138, and PCB-153) indicated one sample whose difference lied outside of the lower and upper limits of agreement, while three outliers can be seen in the Bland Altman plot for PCB-180.

Discussion

Analytic methods

This project has succeeded in creating a highly sensitive and selective method for the detection of PCBs and PBBs in DBS. High extraction recoveries (>90%) were detected for 4 of the 5 analytes, and the calculated accuracies for all 5 analytes were within the acceptable range of 80%-120%. Although correlation analysis indicated that there was a strong positive correlation between methods, it is important to consider that while two methods designed to measure the same variable should have good correlation, high correlation does not always imply that there is good agreement between two methods [28]. The Bland-Altman plots provide a way to assess the bias between the mean differences of two different methods. While Bland-Altman analysis is often considered to be an informal method of analysis, it allowed for the visualization of the data points that lied within 2 standard deviations of the mean difference. In conjunction with the correlation analysis, it can be concluded that the serum and DBS methods have a high level of agreement. Additionally, the consistence seen in the single outliers for PBB-153, PCB-118, PCB-138, PCB-153 (Figures 6-9) may indicate possible problems with a single sample.

As anticipated due to the low sample volume, the S/N ratios for the DBS samples did not reach the standard minimum of 3:1. While the GC-MS/MS analysis of the 65 μL samples resulted in extremely low background noise, the chromatograms (Figures 3-5) displayed acceptable and interpretable peaks. The use of permissive data makes a case for "Fit-for-Purpose" method development. Generally, an understanding of the analytes being measured and their biological relevance can provide a better understanding of the types and limits of data necessary for each individual experiment [29]. Those details being considered, it is then that the decision can be made about whether the use of the DBS method is reliable for the intended application [30, 31]. In every situation, the rigor of method validation is dependent on the purpose of the proposed study. Generally, exploratory method validation includes the consideration of accuracy, precision, selectivity, specificity, recovery and analyte integrity in the matrix, while more advanced validation could include additional specificity, sensitivity, parallelism and method robustness [32, 33]. While the extraction recoveries, accuracies and precisions were shown to be higher when using serum samples, the DBS sample data were comparable, met all standards, and would be considered acceptable for use. While this is true, it is likely that the lower levels of blood extracted from DBS would translate into higher limits of detection than those of serum analysis, and may not be suitable for all purposes. That being considered, if the objective of a study is purely exploratory with a relatively large sample size, the use of DBS analysis may be used as a successful and more cost effective method of analysis, while the more costly serum analysis may be ideal for decision-making or primary objectives [33]. Additionally, DBS analysis would be ideal for studies

wishing to recruit large numbers of children or studies that wish to determine in utero exposures in order to utilize prospective biomonitoring due to the less invasive nature of sample collection and ease of storage after collection.

Limitations

Due to the nature of the pilot study, the sample size was relatively small (N=53) and the analytes were not detectable in every sample. This is especially true for PBB-153, which was detected in 19 of the 53 samples. Future studies utilizing larger sample size may provide a more representative picture of the population and would likely limit the influences of outliers or extreme observations due to variables such as instrumental complications. Additionally, all data collected was based on DBS collected in the field and shipped to Emory University via USPS. In order to determine the level of sample integrity based on shipping method, analysis of DBS shipped using an alternative shipping method such as samples shipped overnight over dry ice should be completed. While it is predicted that the extraction recovery for PCB-118 would fall within the acceptable range, instrumental issues resulted in the inability to definitively determine this information.

Extension to other analytes

Currently, these methods are also being tested for the identification of chemicals such as DDT, DDE, and PBDEs. With minimal adaptations, this method has the potential to be expanded to detect and quantify a multitude of different environmental exposures. This is supported by the method accuracies that range

from 80%-111% with relative standard deviations (RSD) of less than 15%. By utilizing this minimally invasive method for various analytes, scientists may be able to gain knowledge on the degree of various exposures within a population as well as their associated health effects.

Conclusions

The method used in this experiment was adapted from methods developed for the detection of PBBs and PCBs in serum. Due to the PBB incident in Michigan and the ubiquitous nature of PCBs, interest in the effects of environmental exposures has increased over the past 40 years. While this is true, there have been very few advances in analytical methods for these chemicals that would decrease cost and increase participation in studies that help quantify the risks and effects of these environmental exposures. In the past, different methods including the use of GC-ECD, GC-HRMS for the identification of these chemicals have been developed for the detection of PCBs, PBDEs and PCBs in human sera, but each of these comes with their limitations, including the potential for false positives, and high cost, respectively [34-36]. The development of the method using gas chromatography-electron impact ionization-tandem mass spectrometry on human sera provided a highly selective and sensitive approach to congener identification. The adaptation of this method to DBS maintains the selectivity and sensitivity but greatly reduces the invasiveness and cost associated with sampling and analysis. The use of DBS could revolutionize the field of exposure assessment. For the first time, GC-MS/MS analysis of DBS have been shown to provide a cost effective means for the determination of multigenerational health

effects from developmental exposures to various POPs, and unlike serum, this DBS method can be used for prospective biomonitoring.

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Tables and Figures

Table 1: MRM transitions and related parameters by target compound

Target Compound (Ballschmiter-Zell nomenclature)	Retention Time (min)	MRM Transition 1 (parent mass→ fragment mass)	Collision Energy 1 (eV)	MRM Transition 2 (parent mass→ fragment mass)	Collision Energy 2 (eV)
PBB - 153	8.55	467.8 → 307.9	40	627.9 → 467.8	45
PBB-153 (IS)	8.55	479.8 → 319.9	40	-	-
PCB-118	5.90	323.7 → 254.0	30	325.7 → 256.0	25
PCB-138	6.55	359.7 → 289.9	30	359.7 → 279.9	30
PCB-138 (IS)	6.55	371.7 → 301.9	30	-	-
PCB-153	6.30	359.7 → 289.9	40	289.7 → 218.0	40
PCB-153 (IS)	6.30	371.7 → 301.9	40	-	-
PCB-180	7.00	393.7 → 323.9	30	323.7 → 254.0	45
PCB-180 (IS)	7.00	405.7 → 335.9	30	-	-

Table 2: Fortified DBS validation:
In- house material validation including extraction recovery, mean response, accuracy and precision (RSD) of target halogenated biphenyls with additional NIST reference material validation with accuracy and precision (RSD) for applicable target compounds

Target Compound	Spiked Conc. (ng/mL)	Extraction Recovery	Mean Conc. (ng/mL)	Accuracy	RSD	MDL (pg/mL)
PBB-153	0.01	-	0.0098	98.3%	9.3%	40
	0.05	91.8%	0.0541	87.3%	6.0%	
	Certified NIST SRM 1958 (0.421 ± 0.013)	-	0.337	80.0%	-	
PCB-118	0.01	-	0.0108	107.5%	16.4%	28
	0.05	-	0.0552	110.5%	9.6%	
	Certified NIST SRM 1958 (0.412 ± 0.035)	-	0.4375	106.2%	-	
PCB-138	0.01	-	0.0108	108.4%	15.1%	24
	0.05	93.1%	0.0523	104.6%	6.4%	
	Certified NIST SRM 1958 (0.473 ± 0.054)	-	0.3736	81.7%	-	
PCB-153	0.01	-	0.0097	96.5%	11.0%	32
	0.05	93.7%	0.0505	101.0%	2.4%	
	Certified NIST SRM 1958(0.457 ± 0.036)	-	0.4064	85.9%	-	
PCB-180	0.01	-	0.0103	103.2%	15.1%	14
	0.05	90.0%	0.0499	99.9%	8.1%	
	Certified NIST SRM 1958(0.459 ± 0.049)	-	0.3898	84.9%	-	

Table 3: Fortified Serum Validation:
In-house material validation including extraction recovery, mean response, accuracy, and precision (RSD) of target halogenated biphenyls with additional NIST reference material validation with accuracy and precision (RSD) for applicable target compounds

Target Compound	Spiked Conc. (ng/mL)	Extraction Recovery	Mean Conc. (ng/mL)	Accuracy	RSD	LOD (pg/mL)
PBB-153	0.5	97.1%	0.457	91.4%	7.4%	2
	1	-	0.873	87.3%	4.8%	
	Certified NIST SRM 1958 (0.421 ± 0.013)	-	0.397	94.3%	8.0%	
PCB-118	0.5	93.1%	0.468	93.6%	12.8%	1.4
	1	-	0.846	84.6%	3.1%	
	Certified NIST SRM 1958 (0.412 ± 0.035)	-	0.489	118.8%	8.5%	
PCB-138	0.5	99.2%	0.457	91.5%	16.1%	1.2
	1	-	0.858	85.8%	5.0%	
	Certified NIST SRM 1958 (0.473 ± 0.054)	-	0.459	97.0%	3.5%	
PCB-153	0.5	97.2%	0.451	90.1%	9.2%	1.6
	1	-	0.881	88.1%	7.6%	
	Certified NIST SRM 1958 (0.457 ± 0.036)	-	0.441	96.6%	2.8%	
PCB-180	0.5	97.6%	0.448	89.7%	12.5%	0.7
	1	-	0.843	84.3%	6.2%	
	Certified NIST SRM 1958 (0.459 ± 0.049)	-	0.409	89.1%	5.3%	

Table 4: Spearman's correlation analysis using DBS and serum concentrations

Analyte	N	Correlation Coefficient (r)
PBB-153	19	0.93**
PCB-118	52	0.64**
PCB-138	52	0.72**
PCB-153	50	0.75**
PCB-180	46	0.78**

**** Correlation is significant at the 0.05 level.**

Figure 1: General structure of PBBs

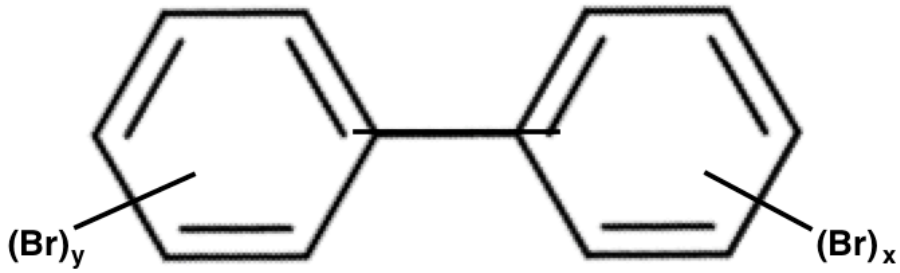

Figure 2: General structure of PCBs

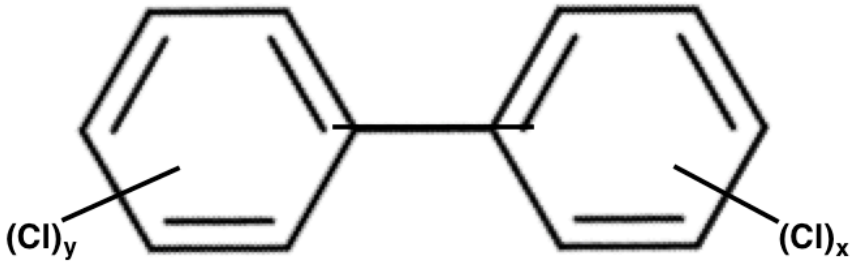

Figure 3: Extracted ion chromatogram of a 0.05 ng/mL equivalent calibrant (S4)

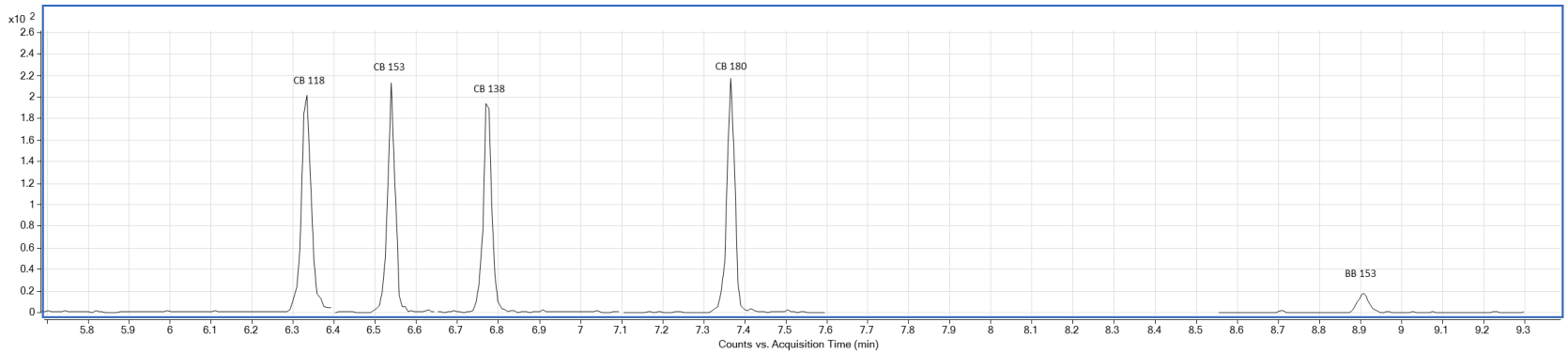

Figure 4: Extracted ion chromatogram of a 0.05 ng/mL fortified in-house DBS (DBS-QCH)

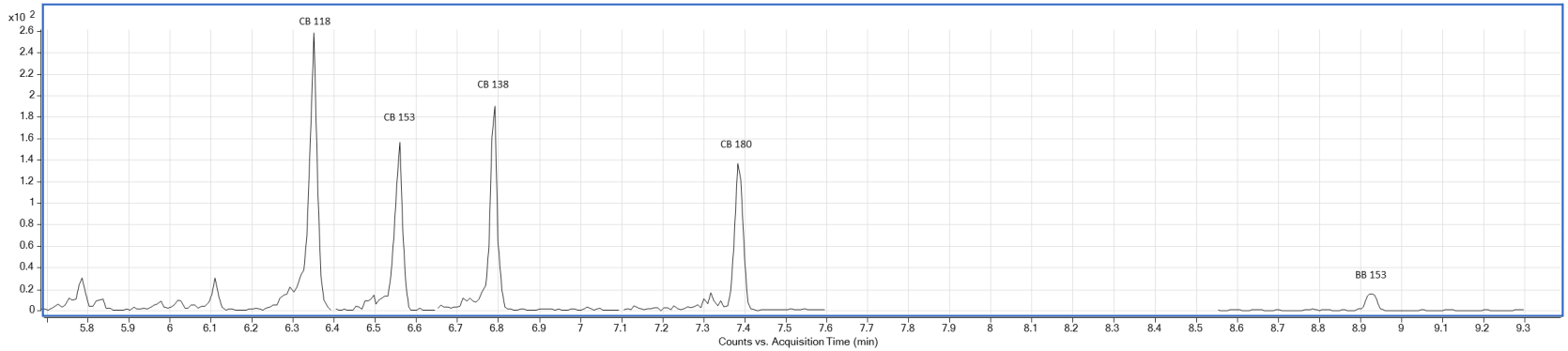

Figure 5-1: Extracted ion chromatogram from unknown DBS sample (99990026)

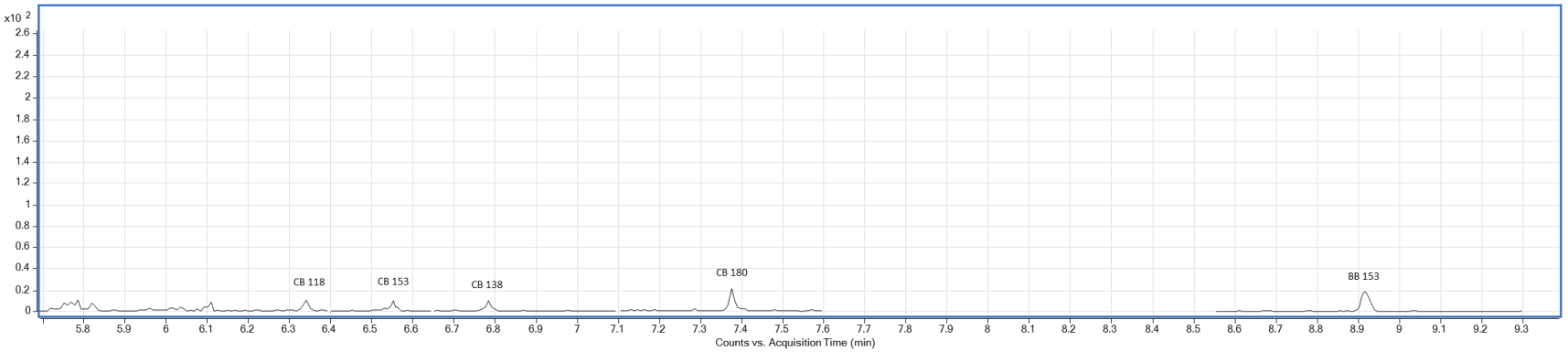

Figure 5-2 : Extracted ion chromatogram from unknown serum sample (99990026)

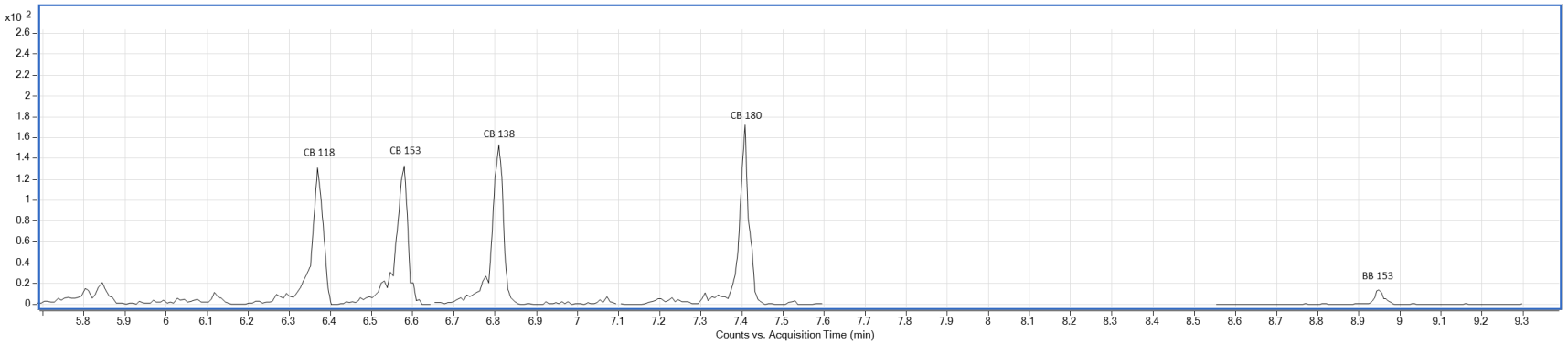

Figure 6: Bland-Altman plot PBB-153

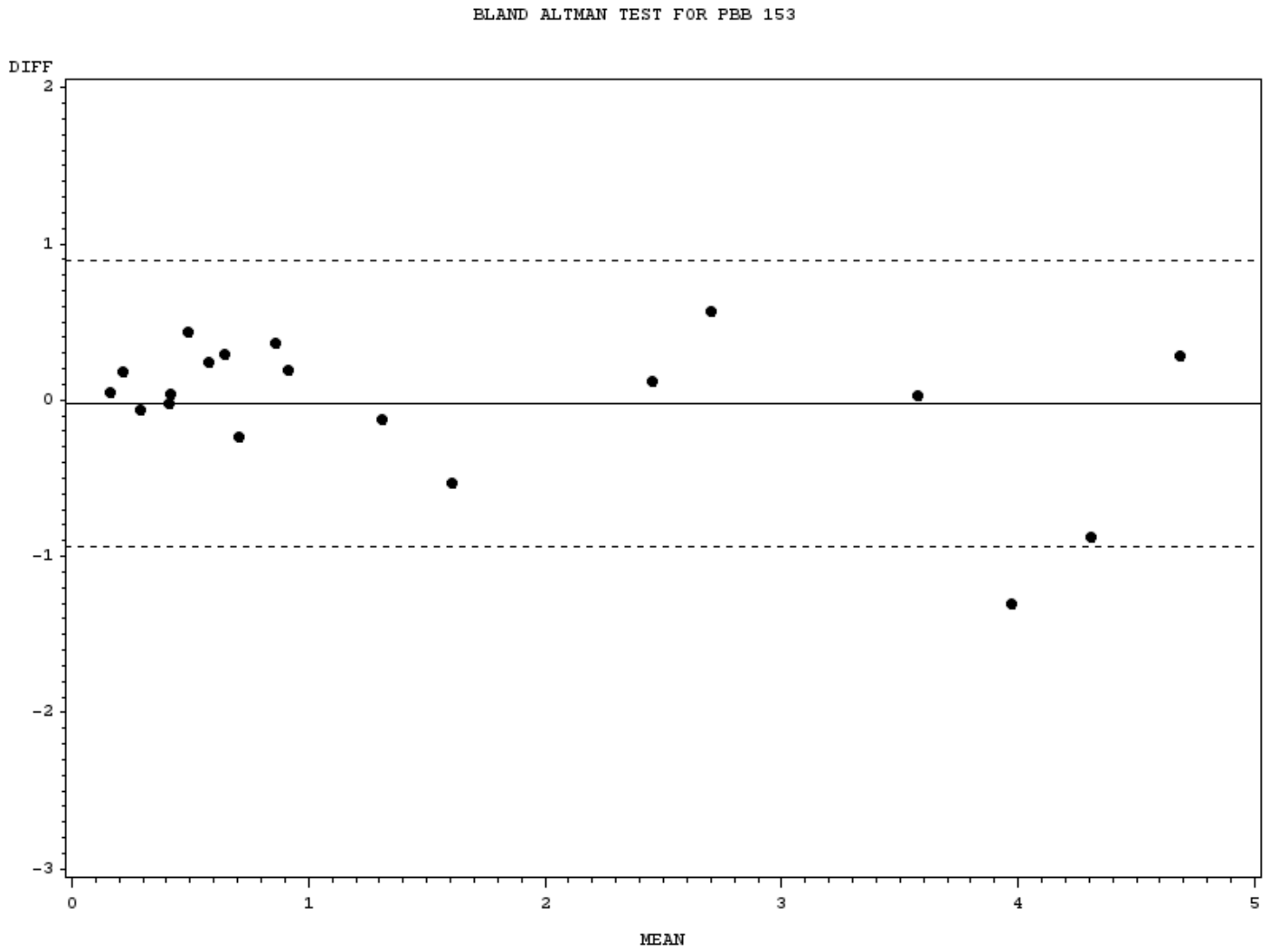

Figure 7: Bland-Altman plot PBB-118

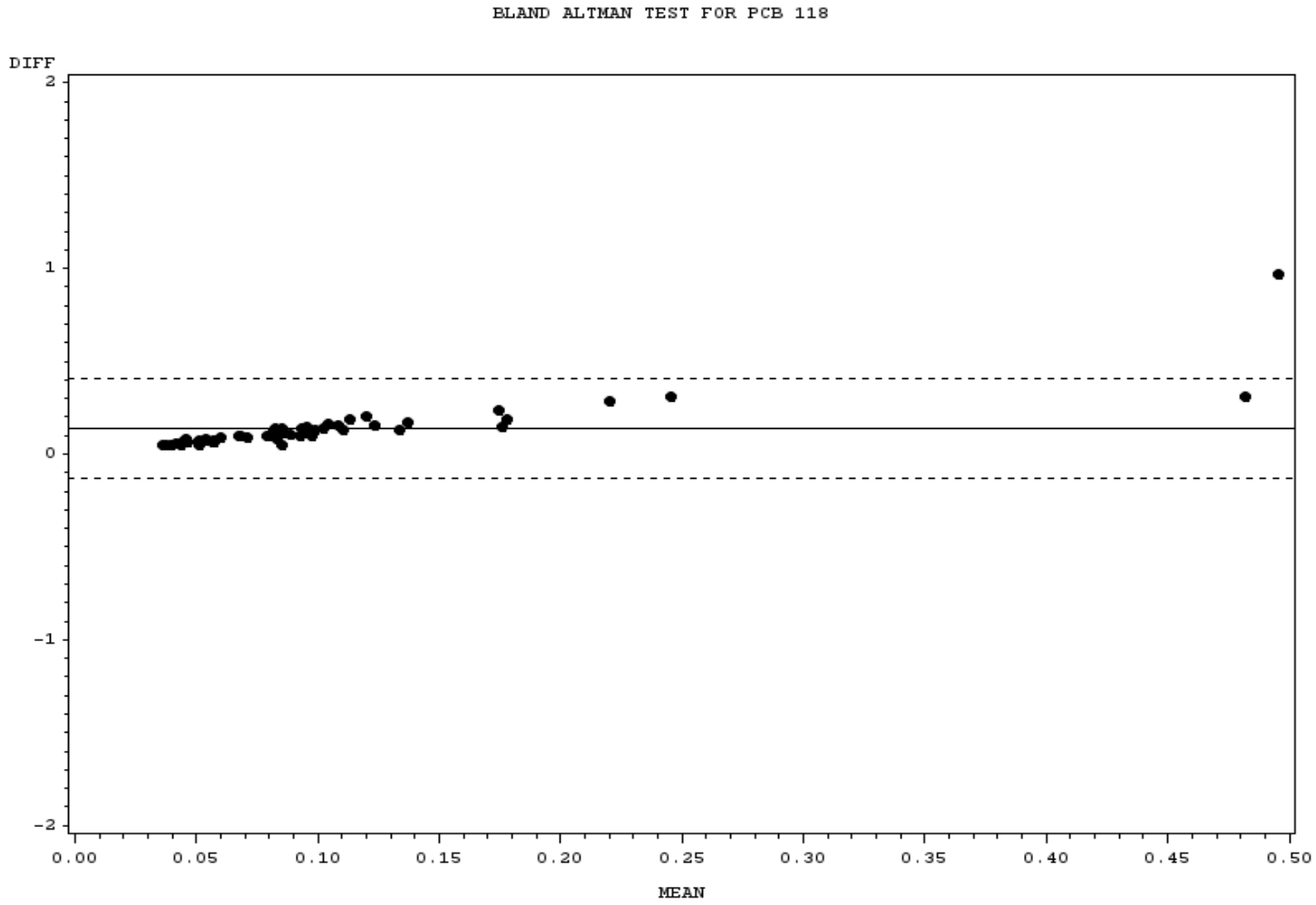

Figure 8: Bland-Altman plot PCB-138

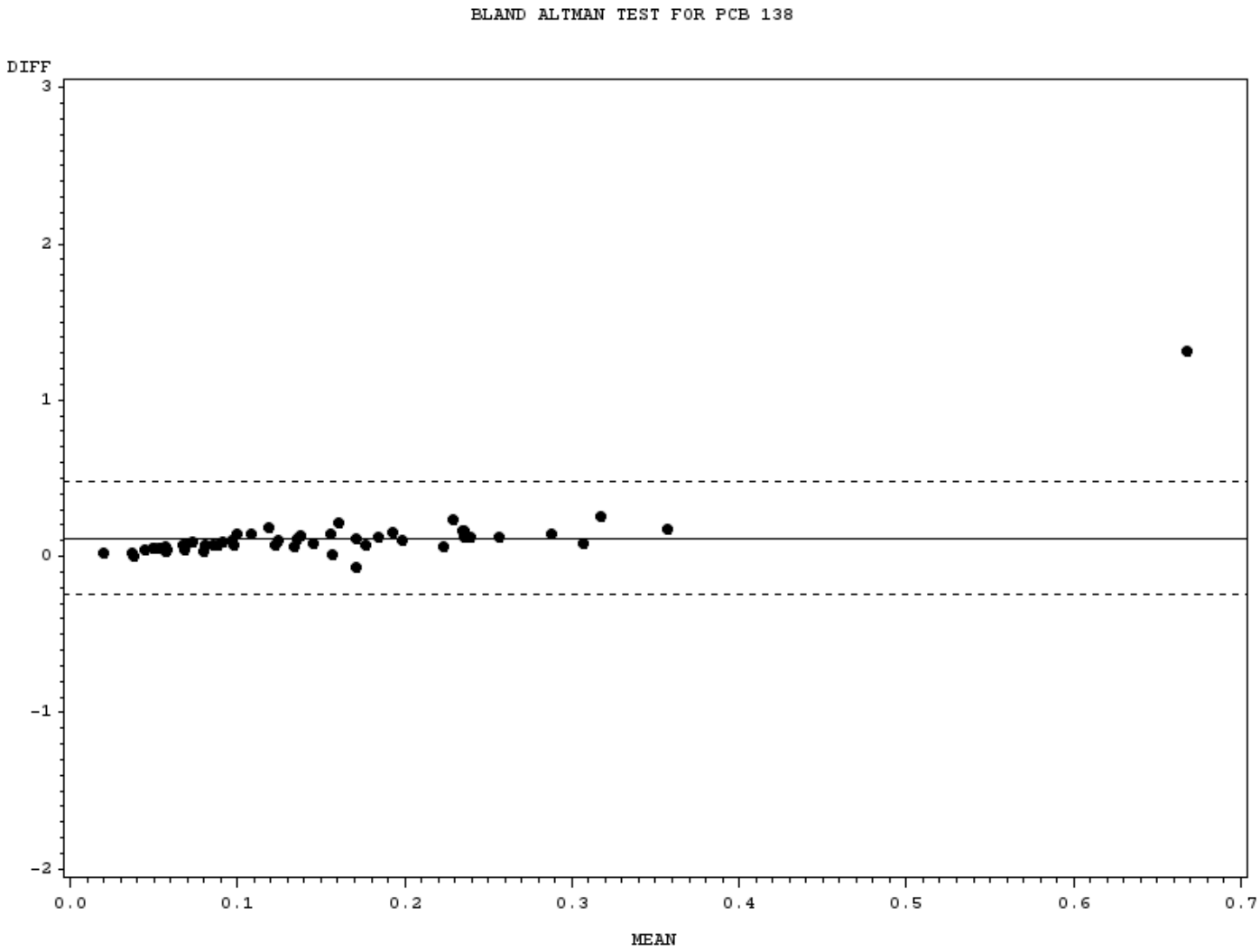

Figure 9: Bland-Altman plot PCB-153

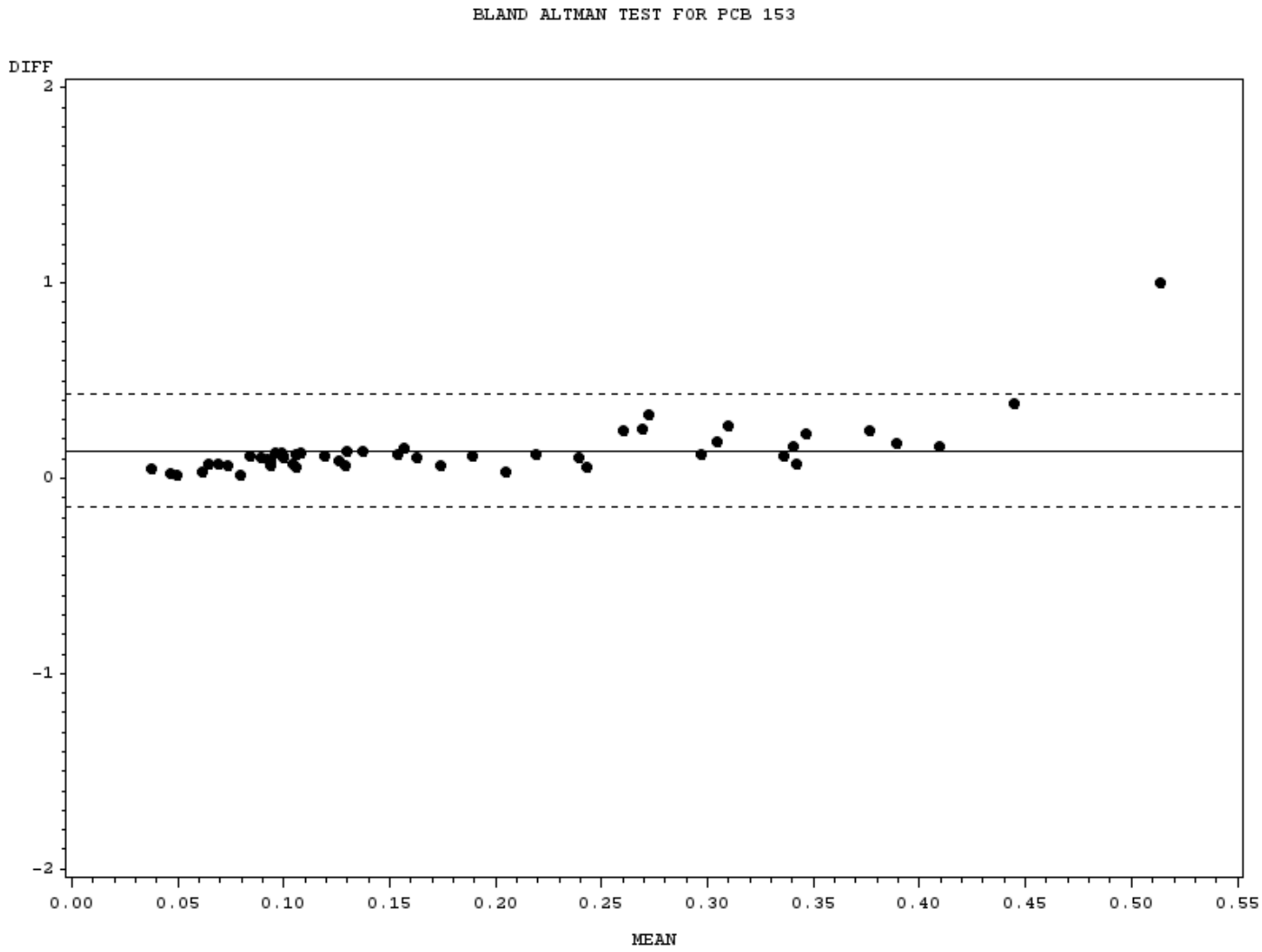

Figure 10: Bland-Altman plot PCB-180