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Validation and Characterization of Dried Blood Spots as an Exposure Matrix for Measuring Persistent Organic Pollutants

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Abstract

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By Anthony Murphy

Persistent organic pollutants (POPs) *p,p*'-dichlorodiphenyltrichloroethane (DDT), *p,p*'-dichlorodiphenyldichloroethylene (DDE), dieldrin, and hexabromobenzene (HBB) are capable of transplacental and lactational transfer, and may pose a particular health risk to the developing fetus. Serum is the traditional biological matrix for assessing exposure to POPs, but has many limitations. Chief among those are the invasive nature of, and expense associated with collection, storage and analysis. Dried blood spots (DBS) are a commonly employed method to measure pharmaceutical and tobacco derivatives in newborns and test for genetic abnormalities such as phenylketonuria. We propose here that DBS can be used to accurately and precisely quantify DDT, DDE, dieldrin, and HBB in newborns. DBS are a relatively non-invasive, cheaper, and more practical alternative to serum for assessing exposure to such toxicants. Employing DBS as a matrix for assessing *in utero* exposure also provides an opportunity for retrospective biomonitoring as well as in assessing multigenerational effects. In order to validate DBS as an exposure matrix, whole blood and DBS samples were collected from a subset (n=52) of the Michigan PBB cohort. GC-MS/MS analysis was used to quantify DDT, DDT, dieldrin, and HBB in both serum and DBS. Method detection limits for DBS were determined to be 0.082, 0.2, 0.312, and 1.93 ng/spot for DDE, dieldrin, DDT, and HBB, respectively. It was found that DBS can be used to accurately and precisely quantify DDT, DDE, and dieldrin (100 ± 20% and <15% RSD). A Bland-Altman assessment for agreement for DDE showed bias for DDE at ± 2SD, and DBS was observed to be higher in value at the latter portion of the Bland-Altman plot. However, serum DDE and quantitated DBS DDE were found to have a high degree of correlation, which demonstrates the potential of this method; correlation coefficient and associated p-value of 0.95 (p-value <0.0001). Based on an estimated costs assessment, DBS is approximately 85 times less expensive than serum using a sample population of 500. Our findings serve to highlight the potential and relevance of DBS as an exposure matrix for POPs such as DDT, DDE, dieldrin, and HBB.

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Introduction

The pesticides *p,p*'-dichlorodiphenyltrichloroethane (DDT) and dieldrin are considered persistent organic pollutants (POPs), and are recognized under the Stockholm Convention's 12 initial POPs as chemicals that cause adverse effects on humans and the ecosystem[1]. The best-known health effect of DDT is nerve impulse conduction, which involves the prolonging of sodium currents in axons, resulting in repetitive after discharges in nerve fibers and synaptic junctions[2]. Respiratory and cardiovascular functions are controlled by the nervous system; therefore, exposure to DDT is expected to result in central and peripheral signs of toxicity[2]. DDT is also known to be a reproductive and developmental toxicant[2]. In particular, DDT and its stable metabolite/degradate, *p,p*'-dichlorodiphenyldichloroethylene (DDE), have been associated with alterations in endpoints controlled by hormonal function such as lactation, maintenance of pregnancy, and fertility[2]. Studies of DDT and DDE have also shown that these chemicals possess carcinogenic properties with the liver being of particular importance[2]. Additional hepatic effects observed in animals exposed to DDT and related compounds include induction of liver enzymes, hypertrophy, hyperplasia, and necrosis[2].

Although results have proven to be contradictory, many efforts have been made to link exposure to DDT/DDE to breast cancer. In one instance, *in utero* exposure to DDT has been liked to breast cancer in a 54-year follow-up study in the Child Health and Development study[3]. While the majority of studies have not found an association between DDT/DDE exposure and breast cancer, other studies have suggested that

DDT/DDE exposure is related to cancer aggressiveness[2]. Demers et al. (2000) linked DDE exposure to increasing mammary tumor size and axillary lymph node involvement, which may be reflective of cancer aggressiveness. Uncovering an association between breast cancer and DDT/DDE is challenging, and factors such as menopause, estrogen receptor status, and exposure to additional compounds that may exert estrogenic or antiestrogenic properties must also be taken into consideration[2].

While the health effects associated with DDT and DDE exposure are vast, it is also of importance to note that transplacental and lactational transfer of DDT and DDE have been reported[5,6]. To investigate the potential birth effects associated with exposure to POPs, Robledo et al. (2015) conducted a prospective pregnancy study, and found that preconception maternal and paternal serum concentrations of POPs were significantly associated with birth size and weight. Significant associations between DDE and crown-heel length, birth weight, and head circumference were also reported, adjusting for gestational age and preterm births[7]. In summary, many of the health effects of DDT and DDE are complex and require further investigation. Quantifying exposure to POPs such as DDT and DDE provides a basis in evaluating health implications, and is of particular importance regarding *in utero* exposure due to the lack of toxicant defense mechanisms in the fetus and the inability to control exposure.

Dieldrin

Like DDT and DDE, aldrin and its structural analogue, dieldrin, are organochlorine pesticides, and were used primarily to control termites, corn pests, and in the citrus industry[8]. However, their use as pesticides on crops such as corn and cotton was discontinued in 1970 under a mandate from the U.S. Department of Agriculture, and

their use in termite remediation was voluntarily canceled in 1987 at the manufacturer's request[9]. Exposure to aldrin and dieldrin typically results from consuming contaminated foods, including crops on which they were sprayed and animal products due to their persistence in the environment and bioaccumulation potential[9]. Exposure to aldrin and dieldrin can also occur via exposure to contaminated drinking water, soil, and air in areas where aldrin and dieldrin persist[9]. Although both aldrin and dieldrin were widely used and their persistence still observed, aldrin is rapidly photolytically and metabolically degraded to dieldrin, making dieldrin of particular interest[9]. Exposure to dieldrin has been reported to result in neurological, hepatic, reproductive, and developmental effects[9]. Like DDT, the primary neurological effects associated with dieldrin is central nervous system excitation and compulsions, and in longer-term exposures some less serious signs of toxicity have been reported and include headaches, dizziness, hyperirritability, and muscle twitching[9]. In animal studies impaired learning, physical signs of neurotoxicity (tremors), and histopathological degenerative changes were observed at doses of 0.1, 0.5, and 0.7 mg/kg/day, respectively[9]. Molecular-based evidence has suggested that dieldrin may block action of $GABA_A$ receptor-chloride channel complex, which may be responsible for some of these effects[9].

Dieldrin's ability to act as a neurotoxicant renders it a cause for concern, especially considering the potential for dieldrin to impair learning. However, decreased fertility following exposure to dieldrin has been reported in animal studies, which adds yet another aspect that must be considered when assessing exposure scenarios[9]. Specifically, there have been reports of toxicity regarding the male reproductive system, and include decreased sperm count, germ cell degeneration, decreased plasma and

testicular testosterone, and decreased plasma luteinizing hormone and follicular stimulating hormone[9].

Lastly, dieldrin has also been found to be associated with developmental effects including external malformations and decreased postnatal survival following *in utero* exposure[9]. These results are controversial in that the types of effects reported were inconsistent with the doses administered, and the mechanism of neonatal lethality is still considered unknown[9]. Dieldrin has been detected in fetal blood, the placenta, and amniotic fluid, indicating that transplacental and translactational transference does occur[10]. This fact paired with neurological, developmental, and reproductive toxicity, calls for further investigation regarding *in utero* and postnatal exposure.

Hexabromobenzene

Brominated flame retardants and in particular, hexachlorobenzene (HBB), are a class of chemicals used in a variety of household products including clothing, textiles, furniture and electronics to inhibit or moderate the burning process[11]. While these compounds may provide an added benefit in preventing or mediating the intensity of household fires, they have been detected in human and environmental media and are considered persistent[12]. This is largely due to the market for these compounds, which in 2001 demand exceeded 67,000 tones according to the Bromine Science and Environmental Forum[13]. In a Tianjin population of Northern China, which is a typical industrial population in the region, HBB was identified in 26 of 115 individuals (23%)[14]. Contrary to this, in 11 pooled breast milk samples from 109 first-time mothers in Ireland, HBB was not detected above the analytical limit of detection (LOD), demonstrating the diversity in magnitude of HBB exposure or demonstrating methodological differences in

LODs[15]. While HBB exposure differs among geographical regions and populations, it is important to investigate the body burden of HBB, considering the potential health impacts that HBB may cause. Although contradictory, it was reported that HBB decreased glutathione (GSH) levels following administration, and over a period of three weeks, increased GSH concentrations[16,17]. In addition, during the first week of exposure to HBB, 5-aminolevulinate synthase (ALA-S) activity was reported to decline, which amounted to approximately 25% across all doses of HBB[16]. Furthermore, in agreement with Carlson (1979) and Smith and Francis (1980), Szymanska and Piotrowski (2000) observed HBB as a porphyrogenic compound based on its ability to increase coproporphyrin and the sum of porphyrins in rats[16,18,19]. As a potential porphyrogenic compound, HBB exposure may result in a dysfunction in the production of hemoglobin, which has been previously associated with skin lesions and infant deaths from cardiorespiratory failure in mothers who were exposed to hexachlorobenzene (HCB), a compound which closely resembles HBB[20].

An assessment of liver mass was conducted to assess potential xenobiotic action in the liver, and a 116% increase in liver mass it was found in rats exposed to HBB relative to control animals[21]. Furthermore, a 10-times difference was observed in CYP1A induction in hepatic microsomes[21]. GSH plays a role in the body's antioxidant defense system, and albeit briefly, a decrease in GSH may have a profound impact on eliminating free radicals, which may result in cellular dysfunction and genotoxic stress[22]. CYP1A also serves an important role in the liver, and is responsible for the metabolism of many xenobiotic including polycyclic aromatic hydrocarbons[21]. It is widely known that CYP1A expression in the fetal liver is relatively low, and that hepatic enzyme expression in the

fetus differs from that of adults[23]. As a result of CYP1A differentiation among fetuses and adults, alternative enzymes may be responsible for xenobiotic biotransformation or elimination, which may prove to enhance or reduce xenobiotic toxicity. Therefore, increased attention in evaluating the presence of compounds such as HBB and potential health effects in newborns is warranted.

Dried Blood Spots (DBS)

Traditionally, serum is the biological matrix commonly employed to assess exposure to POPs; however, its use as a matrix is limited. For instance, serum only provides current body burden estimates, is invasive and presents limitations in using infants and children in such studies, has a high cost associated with collection and transfer of samples, and may only provide retrospective biomonitoring if previously collected and achieved correctly[24]. More recently, however, the application of DBS as a matrix for assessing newborns' exposure to environmental toxicants has been of interest. DBS are a unique biological matrix commonly used in the newborn screening process consisting of identifying genetic abnormalities such as phenylketonuria, sickle cell disease and hypothyroidism, and exposure to pharmaceutical drugs and tobacco derivatives in newborns [24,25,26]. However, by using DBS as a matrix for assessing exposure to POPs such as DDT, DDE, dieldrin, and HBB, multigenerational health effects can be evaluated. In addition, DBS can be used to assess temporal trends of environmental toxicants and in environmental epidemiology studies evaluating newborns exposure levels and health outcomes. Therefore, assessing DBS as an exposure matrix to quantify exposure to environmental toxicants in infants and children is worthwhile. The purpose of this thesis is to evaluate the reliability of DBS as a matrix for assessing

exposure to dieldrin, DDT, DDE, and HBB, and its comparability to traditional serum measurements using samples collected from chemical workers and their families. We hypothesize that we can accurately (100 ± 20%) and precisely (relative standard deviation <15%) measure DDT, DDE, dieldrin, and HBB in dried blood spots, and that the concentrations obtained from dried blood spots will be highly correlated with those obtained from serum sample equivalents. Alternatively, concentrations of DDT, DDE, dieldrin, and HBB will not be measurable at 100 ± 20% accuracy and <15% precision, and will not be correlated for serum and DBS quantification methods.

Materials and Methods

Study Population

In the 1970s a nutritional supplement for livestock feed under the trade name Nutrimaster® was inadvertently substituted with Firemaster®, a brominated flame retardant containing poly brominated biphenyls (PBBs). As a result of this inadvertent substitution, Michigan residents were primarily exposed to PBBs by consuming animal-derived products including beef, poultry, and dairy products. In addition to this, from 1938 to 1978 industrial activities of Velsicol Chemical Corp. (formerly Michigan Chemical Corp.) led to widespread contamination in the surrounding area including: a residential area, municipal water supply, three EPA superfund sites, and Pine River, which borders the site of the former chemical plant[27,28]. The Michigan Department of Community Health (MDCH) established a PBB registry, which included those exposed to PBBs and a subset of chemical workers and their families. However, in 1990 the chemical workers and their families were dropped from the registry because it was thought that their exposure to multiple chemicals (e.g., DDT, DDE, dieldrin, and HBB)

could not be properly addressed at the time. Both the residents of Pine River and Velsicol chemical workers are concerned about both their exposure to PBBs as well as industrial chemicals, and after receiving a request from the Pine River Superfund Citizen Taskforce, Mid-Michigan District Health Department, and PBB Citizen Advisory Board, the Emory PBB Research Team was able to obtain support and include this population in the ongoing Michigan PBB Research Registry. A subset of 52 of these chemicals workers agreed to participate in our DBS validation study by providing both serum and DBS samples.

Chemicals

Acetonitrile, hexane, isopropanol, methanol (analytical grade), and Florisil cartridges were purchased from Thermo Fisher Scientific (Waltham, MA). Ethyl acetate and sodium sulfate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), and toluene was purchased from Avantor Performance Materials (Center Valley, PA). Water was generated using Milli-Q Ultrapure water purification system (Millipore, Billerica, MA). C-18E cartridges were purchased from Phenomenex (Torrance, CA).

Whatman 903 protein saver cards were obtained from VWR International (Radnor, PA). Whole blood for preparation of blood spot matrix quality controls was drawn in-house, and pooled human sera was obtained from the Red Cross (Interstate Blood Bank, LLC; Memphis, TN). Standard reference material (SRM 1958, organic contaminants in fortified human sera) was purchased from the National Institute for Standards and Technology (NIST, Gaithersburg, MD). Helium and Nitrogen, 99.999% ultra-high purity, were purchased from nexAir, Inc. (Suwanee, GA). The purity of all native standards was ≥98%. Dieldrin, HBB, DDT, and DDE were purchased from

Cambridge Isotope Laboratories (Andover, MA), as individual congeners at 100 ng/mL concentrations in nonane or toluene. A HBB ^{13}C-ring labeled standard (99% purity) at 100 µg/mL in toluene, and DDE and DDT ^{13}C-ring labeled standards (99% purity) at 100 µg/mL in nonane were purchased from Cambridge Isotope Laboratories.

Standards and Quality Control

Native DDT, DDE, dieldrin and HBB mixed calibration standards were prepared by serial dilution of 0.05 ng/µL stock solution in acetonitrile:toluene (3:1). All calibrants were solvent-based standards with each concentration expressed in serum-equivalents. These were prepared separately for serum and blood spot analyses. The highest calibrant concentration was equivalent in to that in the final extract from a serum sample whose concentration was 5 ng/mL. By expressing the concentrations in serum-equivalents, our overall quantification scheme was simplified. Calibration standards serum equivalent concentrations ranged from 0.01 ng/mL across 9 points for both serum and blood spots. A labeled standard spiking solution was prepared at a concentration of 0.4 ng/mL in acetonitrile:toluene (3:1).

Four quality control (QC) spiking solutions containing the native mixture were prepared with standard spiking solutions by serial dilution of the initial stock solution, two for use with serum matrix and two for use with blood spot matrix. When spiked into the serum matrix, the nominal concentrations of these matrix-based QC samples were 0.05 and 1.0 ng/mL. When spiked into the blood spot matrix, the nominal concentrations of these matrix-based QC samples were 0.01 ng/mL and 0.05 ng/mL. All standard stock solution and spiking solutions were dispensed into amber vials and stored at 4°C until

used. Whole blood was drawn from 1 individual for QC blood spots, which were uniformly 65 µL.

Extraction

Each blood spot sample (approximate blood volumes range from 50 µL to 75 µL) was spiked with 25 µL labeled standard solution resulting in 5 ng/mL internal standard concentration. Samples were then vortex mixed briefly before adding 2 mL of 5% sodium sulfate water: propanol solution (85:15). Each sample was then vortex mixed at 2000 rpm for 5 minutes using a multivortexer (Benchmark BenchMixer, Edison, NJ), and then sonicated for 5 minutes. Following this step, each sample was again vortex mixed for an additional 5 minutes. Sample extraction was performed using C18-E cartridges with clean-up performed with Florisil cartridges. Conditioning for C18-E cartridges was performed using 3 mL methanol followed by 3 mL of 5% sodium sulfate water: propanol solution (85:15) immediately prior to sample extraction. Samples were loaded onto the cartridges; sample breakthrough was not collected. Sample cartridges were washed with an additional 3 mL of 5% sodium sulfate water: propanol (85:15), which was not collected. Sample elution step used 10 mL hexane: ethyl acetate (1:1), with the eluate collected in clean labeled test tubes. The aqueous layer was subsequently removed from the eluate. 50 mg of sodium sulfate was added to test tubes to ensure separation of the organic layer. Samples were vortex mixed at 2000 rpm for 5 minutes using a multivortexer, and then centrifuged prior to decantation of the organic layer into a new labeled test tube. Test tubes containing the isolated organic layer were inserted into the TurboVap® (Zymark, Framingham, MA) set at 45°C and 20 psi and evaporated for 4 minutes to reduce the total solvent volume to 3 mL. Samples were then loaded onto

Florisil cartridges conditioned with 5 mL hexane: ethyl acetate (1:1), and breakthrough was collected. The cartridges were eluted with 10 mL hexane: ethyl acetate (1:1), with eluate collected in the same test tube as the breakthrough, which were then evaporated to total dryness using TurboVap®. Samples were reconstituted with 20 µL of acetonitrile : toluene (3:1) for instrumental analysis.

Instrumentation and Analysis

Analysis was performed using an Agilent 7890A gas chromatograph coupled to an Agilent 7000B tandem mass spectrometer (Agilent Technologies, Santa Clara, CA). The system was controlled using MassHunter Workstation Software version B.05.00. Calibration and instrument tuning was performed in EI with High Sensitivity Autotune mode, and instrument performance was always checked prior to analysis. The GC system used a 5% phenyl methylpolysiloxane analytical column (30 mm x 0.25 ID x 0.25 internal film thickness, HP-5MS, Agilent Technologies) for optimum separation. 2 µL injection volume was used with injection port temperature at 250°C under splitless mode. The GC helium carrier gas flow rate was 1.2 mL/min through the end of the run. The oven temperature program was as follows: 90°C (0.1 min), increased at 40°C/min to 200°C and held for 1 min, increased at 40°C/min to 220°C and held for 7.2 min, and then increased at 30°C/min to 320°C and held for 2.25 min. The total run time was 17.1 min. Source and quadrupole temperatures were set to 230°C and 150°C, respectively.

For the MS/MS quantification method, individual injections of each target compound in full scan mode was performed to obtain retention times, and to select proper precursor ions, generally selecting the most intense ion with the highest *m/z* ratio. Product ion scan was performed using different collision energies set to determine the most

selective precursor ions. Ions were selected based on their intensity, peak shape, and signal-to-noise ratio. Two transitions were selected for each native analyte for quantification and confirmation. Only one transition was selected for each labeled analyte, ^{13}C-*p,p*-DDE, ^{13}C-*p,p*-DDT, and ^{13}C-HBB. All transitions were monitored in multi-segment analysis using multiple reaction monitoring (MRM) mode. These MRM transitions and associated parameters are described in Table 1.

Data Processing

Data were processed using MassHunter Workstation Software – Quantitative Analysis version B.05.00. Each compound was characterized by its retention time, signal-to-noise ratio, relative retention time value, peak algorithm, quantitation transition, and confirmation transition.

Chromatographic Separation

Unfortified in-house DBS and Red Cross pooled serum (which served as method 'blank' samples) showed no isobaric or chromatographic interferences with target compounds. Figure 1.1 shows the extracted ion chromatograms for target native compounds from analysis of 0.05 ng/mL calibrant, a typical 0.05 ng/mL fortified in-house DBS sample, a typical 0.05 ng/mL fortified in-house pooled serum sample, and an unknown dried blood spot and paired serum samples.

Extraction Recovery of Dried Blood Spots

DBS extraction recoveries are presented in Table 2. Target compounds have a recovery range of 29.8% to 39.3% using the OC serum extraction method previously described.

Duplicate Analysis

Duplicate analyses were performed for DBS, and percent agreement for DDE was determined to be 99%. Dieldrin, DDT, and HBB were not detected, however.

Serum Equivalent Calculations

Serum comprises approximately 40% of whole blood, which corresponds to approximately 26 μL in DBS. In order to express DBS concentrations as serum equivalents, a correction factor of 38.5 was used to compensate for this difference.

Limit of Detection

Traditionally, LODs are calculated on the basis of a signal-to-noise (SN) ratio of 3. However, since DBS have a very low volume of blood, the noise in the chromatograms is essentially non-existent resulting in discernable peaks with low or no calculated SN ratios. Consequently, 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 the concentration was measureable in a corresponding standard solution. Thus, the LODs represented here are more technically a minimal detectable level (MDL) because its designation is not based upon an objective analytical process.

Calculation of MDL

Based on the lowest concentration obtained with a SN of 1 and acceptable accuracy, DDE, dieldrin, DDT, and HBB were determined to have estimated MDLs corresponding to 0.082, 0.2, 0.312, and 1.93 ng/spot, respectively. The estimated MDL for dieldrin, was calculated by using the QCL (conservative estimate; 0.1 ng/mL) since this was the lowest obtained concentration with a SN estimate of 1 and acceptable

accuracy. To obtain the estimated MDL per spot, concentrations observed with a SN of 1 and acceptable accuracy were multiplied by the reconstitution volume; 20 µL.

Results

DBS Accuracy and Precision

Accuracy was assessed by taking the blank adjusted observed concentration as a percent of the expected. Expected concentrations were 0.01 ng/mL for QCL and 0.05 ng/mL for QCH samples. Precision (relative standard deviation; RSD) for QCL and QCH samples was calculated using the uncorrected standard deviation as a percentage of the average of the blank corrected observations. Both the accuracy and RSDs for QCL and QCH samples for each analyte is presented in Table 2. The accuracy ranged from 81.1% to 103.4% at both fortified levels, and the RSDs ranged from 0.9% to 17.2%. However, the lower level fortified HBB was below the limit of detection, thus there were no attainable accuracy or precision estimates for this level. Both accuracy and precision results meet the United States Food and Drug Administration (FDA) bioanalytical method development recommended criteria, which are 80-120% for accuracy and <20% for precision. These guidelines were also met for NIST SRM 1958 reference samples, which ranged from 82.3% to 108.19% in their accuracy and 0.5% to 4.2% in their precision (Table 2.).

Serum Accuracy and Precision

Serum accuracy was determined as the blank adjusted observed concentration as a percent of the expected. Expected concentrations were 0.5 ng/mL for QCL and 1.0 ng/mL for QCH. RSDs for serum QCL and QCH samples was determined as the uncorrected standard deviation as a percentage of the average of the blank corrected observations.

Accuracy and RSDs for serum are presented in Table 3. The accuracy and RSDs for both fortified levels ranged from 87.47% to 119.37% and 0.2% to 3.7%, respectively. Accuracy and precision for both sets of serum samples fortified with 0.5 ng/mL and 1.0 ng/mL abide by the FDA recommended criteria of 80-120% for accuracy and <20% for precision.

Percent Agreement

Descriptive analysis using a histogram with bin midpoints of 50% was performed to determine the distribution of observations in percent agreement between serum DDE and quantitated DBS DDE concentrations (Figure 1.2). 86.54% of the observations in percent agreement reside between 75% and 225%, which encompasses the majority of the observations. 3.85% of the observations were below 75%, and 9.26% of the observations were above 225%.

Correlation Analysis

Pearson correlation analysis was conducted for serum DDE and quantitated DBS DDE. Exposure age and blood draw age correlations were also explored since it is well documented that with increasing age there is generally an increase in DDT/DDE body burden, although not the primary aim of this thesis. Results are presented in Tables 4-6, and correlation graphs are depicted in Figure 1.3.

For serum DDE and quantitated DBS DDE the correlation coefficient and associated p-value was 0.95 (p-value <0.0001). For blood draw age correlations, the correlation coefficient and associated p-values were 0.47 (p-value = 0.0004) for serum DDE, 0.03 (p-value = 0.89) for serum DDT, and 0.43 (p-value = 0.0017) for quantitated DBS DDE. Lastly, the correlation coefficients and associated p-values for exposure age

were 0.56 (p-value <0.0001) for serum DDE, 0.12 (p-value = 0.57) for serum DDT, and 0.52 (p-value <0.0001) for quantitated DBS DDE.

To determine the most appropriate correlation analysis, outliers were assessed for each of the variables serum DDE, serum DDT, and quantitated DBS DDE. Using the Tukey fence approach, serum DDE was determined to have 1 outlier, quantitated DBS DDE was determined to have 3 outliers, and serum DDT had no outliers. Although there were a limited number of outliers and the majority of these outliers were within 1 SD of the Tukey fence limits, Spearman correlation analyses were conducted, and results are presented in Tables 7, 8, and 9.

The Spearman correlation for serum DDE and quantitated DBS DDE was 0.94 (p-value <0.0001). For exposure age correlations, the Spearman correlation coefficients and associated p-values were 0.50 (p-value = 0.0001) for serum DDE, 0.07 (p-value = 0.74) for serum DDT, and 0.47 (p-value = 0.0004) for quantitated DBS DDE. Likewise, for blood draw age, the Spearman correlation coefficients were 0.48 (p-value = 0.0003) for serum DDE, 0.10 (p-value = 0.66) for serum DDT, and 0.45 (p-value = 0.0009) for quantitated DBS DDE.

Bland-Altman Assessment for Agreement

A Bland-Altman analysis was used to assess the level of agreement between serum and DBS methods (Figure 1.4). A range of agreement was defined as a mean bias of ± 2 SD.

Cost Assessment

A cost assessment was conducted to compare both serum and DBS sampling and analysis (Tables 10 and 11, respectively). Estimates were determined on a per sample

basis for simplification and clarity. Material costs were obtained by a general internet search, and the lowest value observed was used. To provide a more realistic idea of costs associated with a study using either serum or DBS, a sample size of 500 was considered in the assessment.

Correlation Maximization

In order to assess the best possible correlation between serum DDE and quantitated DBS DDE and determine the value above which quantitated DBS DDE is most correlated with serum DDE, a series of correlations are presented. The baseline correlation is the original correlation presented in Table 6. Each subsequent correlation was constructed by removing the bottom 5%, 10%, 25%, and 30% of quantitated DBS DDE observations. Both Pearson and Spearman correlations are presented in Table 13. along with the original correlation. Overall, there was a consistent decrease in correlation coefficients after removing the bottom 5%, 10%, 25%, and 30% of DBS observations. This suggests that there exists no DBS concentration above which the correlation between serum DDE and quantitated DBS DDE is maximized, therefore the original correlation coefficient is the best estimate of association.

Discussion

This study demonstrates that DBS can be used as an exposure matrix to accurately and precisely quantify DDT, DDE, and dieldrin. HBB was not detected at the lower fortified level, however. With the exception of HBB at the lower fortified level, all compounds at both high and low fortified levels met the accuracy and precision criteria of 100 ± 20% and <15%, respectively. Furthermore, our hypothesis that the concentrations obtained from DBS will be highly correlated with those obtained from serum sample

equivalents held true for DDE. The correlation between serum DDE and quantitated DBS DDE and associated p-value was 0.95 (p-value <0.0001). However, in DBS samples, DDT, dieldrin, and HBB were not detected, therefore correlations were not determined for these compounds.

In addition to the correlation between serum DDE and quantitated DBS DDE, it was found that both serum DDE and quantitated DBS DDE were moderately correlated with blood draw age, 0.47 (p-value = 0.0004) and 0.43 (p-value = 0.0017), respectively. Exposure age was also moderately correlated with both serum DDE and quantitated DBS DDE, 0.56 (p-value <0.0001) and 0.52 (p-value <0.0001), respectively. These correlations provide evidence that suggests with increasing age there is increasing body burden of DDE. Many studies have observed increasing concentrations of POPs with increasing age, which is in agreement with the results presented here[29,30,31]. However, because of biotransformation differences between males and females, the body burden in females often declines or remains constant[29,30,31]. Reproductive parameters such as nursing duration and lactation rates may help explain these differences[32]. There may also be differences in biotransformation between POPs such as DDT, DDE, dieldrin, and HBB due to factors such as enzyme kinetics, partition coefficients, and phase I and II metabolic pathways. Therefore, to more accurately describe body burden-age trends, factors such as these should be considered.

The Bland-Altman assessment for agreement showed bias for DDE at ± 2SD. Overall, there was an increasing trend, which starts to flare at an approximate average of 0.2 ng/mL. This suggests that DBS is higher at the high end or lower at the low end. In future studies this should further be explored and bias minimized if possible.

The ability to utilize DBS as an exposure matrix has many potential advantages compared to traditional method for quantifying persistent organic pollutants. The advantages in using DBS are presented in Table 12., demonstrating why DBS are more favorable. In addition to the practical advantages of using DBS as opposed to serum, the cost analysis conducted provides further support for the use of DBS. Based on the estimated costs, DBS is approximately 85 times less expensive than serum analyses. Although this analysis is estimated and costs many vary, the estimate provided here is a conservative estimate. Purchasing DBS and blood lancets in bulk may further reduce the costs. Furthermore, postage was estimated as the current costs per stamp per DBS sample. A more likely scenario would involve shipping multiple DBS cards together, thereby reducing costs even further.

Limitations

One particular concern in this study includes the volume of blood used for DBS spotting (approximately 50-75 μL). While using such a small volume of blood is preferable, replication may become an issue, therefore is critical for further investigation. Extraction recoveries in this experiment ranged from 29.8% to 39.3%, and may reflect adhesion to DBS cards and glass vials. Also, the methods here were optimized for multiple suites of chemicals, which may result in lower than anticipated recoveries. The inability to detect of HBB at the lower fortified level in DBS may have occurred as a result. Therefore, analytical methods should be developed with this in mind.

Conclusions and Future Directions

DBS may be able to be used with contemporary biomarkers of exposure to environmental toxicants in future studies. DBS may also be best suited to qualitative

assessments, depending on the compounds of interest. Aside from this, DBS require improved methods of detection for different suites of compounds. In particular, to measure HBB a more selective analytical method may be required, and this may hold true for additional environmental toxicants. To improve MDLs multiple DBS may be used, and should be explored as a potential solution to overcome these limitations. While this study provided a snap shot of exposure for individuals in the study population, DBS may also be used to assess longitudinal body burden-age trends. This would help provide more information on the biotransformation and accumulation of POPs in humans, which is especially important in the case in women of reproductive age. Lastly, one of the major benefits in using DBS includes the potential to assess multigenerational effects. While there is still much work to be done with DBS, this aspect may prove to be unique and invaluable, and exploring the use of DBS for this purpose is of particular interest in the context of quantifying environmental toxicants in newborns.

Funding

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Tables and Figures

Table 1.

Analytical method parameters

Target Compound	Time Segment	MRM Transition 1	Collision Energy 1 (eV)	MRM Transition 2	Collision Energy 2
Dieldrin	2	262.7 → 193	35	79 → 51	30
p,p-DDE	2	245.9 → 176	35	247.9 → 176	30
p,p-DDE (IS)	2	258 → 188	35	-	-
p,p-DDT	4	234.9 → 199	15	234.9 → 165	25
p,p-DDT (IS)	3	247 → 177	25	-	-
HBB	5	552 → 392	35	472 → 392	30
HBB (IS)	5	558 → 479	30	-	-

Table 2.

Accuracy and RSDs for QCL and QCH samples and certified NIST SRM for DDE, dieldrin, DDT, and HBB

Target Analyte	Fortification (ng)	Extraction Recovery	Mean concentration (ng/mL equivalent)	Accuracy	RSD
***p,p*-DDE**	0.01/DBS	-	0.0083	82.60%	0.90%
	0.05/DBS	34.97%	0.0458	91.53%	5.70%
	Certified NIST SRM 1958 (1.250 ± 0.130)	-	0.4078	80.67%	2.30%
Dieldrin	0.01/DBS	-	0.0081	81.08%	10.50%
	0.05/DBS	39.25%	0.0495	99.03%	2.30%
	NIST SRM 1958, spiking concentration of 500 pg/mL	-	0.541	108.19%	0.50%
***p,p*-DDT**	0.01/DBS	-	0.0103	103.44%	6.90%
	0.05/DBS	34.39%	0.0453	90.57%	5.30%
	Certified NIST SRM 1958 (0.293 ± 0.012)	-	0.2414	82.34%	0.50%
HBB	0.01/DBS	-	<LOD	N/A	N/A
	0.05/DBS	29.83%	0.0431	86.25%	17.20%
	NIST SRM 1958, spiking concentration of 500 pg/mL	-	0.4549	90.97%	4.20%

Table 3.

Accuracy and RSDs for serum

Target Analyte	Fortification (ng)	Mean concentration (ng/mL equivalent)	Accuracy (%)	RSD (%)
***p,p*-DDE**	0.5	0.4374	87.47	0.50
	1.0	0.9490	94.90	0.50
Dieldrin	0.5	0.5326	106.51	3.30
	1.0	1.1937	119.37	0.40
***p,p*-DDT**	0.5	0.4993	99.85	1.00
	1.0	0.9611	96.11	3.40
HBB	0.5	0.5019	100.39	0.20
	1.0	1.1623	116.23	3.70

Table 4.

Pearson Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	BD_Age
SerumDDE	0.47074
SerumDDE P-value	0.0004
***SerumDDT_Adjusted**	0.02864
SerumDDT_Adjusted P-value	0.8968
Quantitated DBSDDE	0.42509
Quantitated DBSDDE P-value	0.0017
***SerumDDT n=23**	

Table 5.

Pearson Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	Exp_Age
SerumDDE	0.55685
SerumDDE P-value	<.0001
SerumDDT_Adjusted	0.12463
SerumDDT_Adjusted P-value	0.571
Quantitated DBSDDE	0.51705
Quantitated DBSDDE P-value	<.0001
***SerumDDT n=23**	

Table 6.

Pearson Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.94579
Quantitated DBSDDE P-value	<.0001

Table 7.

Spearman Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	Exp_Age
SerumDDE	0.50344
SerumDDE P-value	0.0001
SerumDDT_Adjusted	0.07277
SerumDDT_Adjusted P-value	0.7414
Quantitated DBSDDE	0.47014
Quantitated DBSDDE P-value	0.0004
***SerumDDT n=23**	

Table 8.

Spearman Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	BD_Age
SerumDDE	0.47841
SerumDDE P-value	0.0003
SerumDDT_Adjusted	0.09585
SerumDDT_Adjusted P-value	0.6635
Quantitated DBSDDE	0.44605
Quantitated DBSDDE P-value	0.0009
***SerumDDT_Adjusted N=23**	

Table 9.

Spearman Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.9399
Quantitated DBSDDE P-value	<.0001

Table 10.

Estimated Serum Costs	**U.S. Dollar ($)**
[a]Phlebotomist	5.00
Vacutainer	10.00
Dry Ice	5.00
Shipping	20.00
Ancillary	20.00
[b]Total	60.00
Total for 500 person study	30000.00
[a]Blood draw per person by trained phlebotomist is estimated as 15 minutes	
[b]Per sample	

Table 11.

Estimated Dried Blood Spot Costs	**U.S. Dollar ($)**
DBS card	0.20
[a]Blood lancet	0.02
Postage (mailed individually)	0.49
[b]Total	0.71
Total for 500 person study	352.50
[a] Blood lancet is self-administered	
[b]Per sample	

Table 12.

DBS Vs. Serum as an Exposure Matrix for Persistent Organic Pollutants	
Advantages	**Potential Disadvantages**
Ability to collect multiple spots, and decrease MDL	MDL challenges
Can use spots that have not been tampered with for additional analyses	Variability in volume of blood spotted
	May be difficult to manipulate in laboratory settings
Potential for be used in retrospective studies	
Cost efficient	
Serum requires separation from whole blood, which adds to sample preparation time	
Can be used to assess multigenerational effects	
Potential to evaluate temporal trends	
Reflects newborn exposure	
Readily collected during newborn screening process	
Serum needs to be thawed, which can cause chemical degradation	
Serum can only be used for retrospective studies if stored correctly	
Serum only provides current body burden estimates	
Serum is invasive, which presents limitations in assessing newborn exposure	

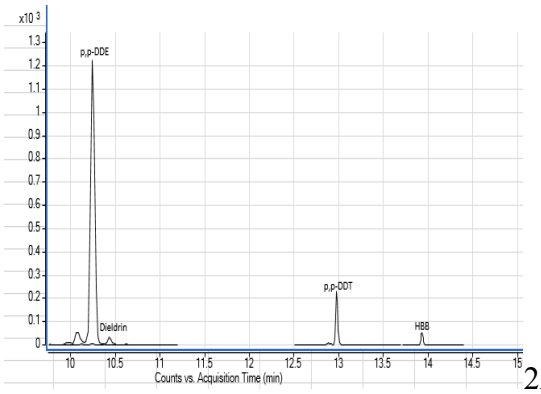
Table 13.

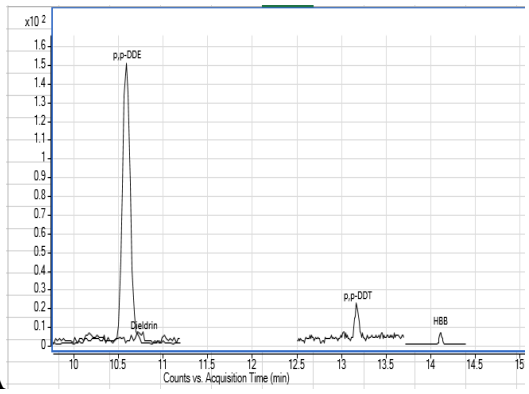
Without removing any observations	
Pearson Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.94579
Quantitated DBSDDE P-value	<.0001
Spearman Correlation Coefficients, N = 52	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.9399
Quantitated DBSDDE P-value	<.0001
After removing bottom 5%	
Pearson Correlation Coefficients, N = 49	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.94209
Quantitated DBSDDE P-value	<.0001
Spearman Correlation Coefficients, N = 49	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.92827
Quantitated DBSDDE P-value	<.0001
After removing bottom 10%	
Pearson Correlation Coefficients, N = 45	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.93701
Quantitated DBSDDE P-value	<.0001
Spearman Correlation Coefficients, N = 45	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.91357
Quantitated DBSDDE P-value	<.0001
After removing bottom 25% Q1	

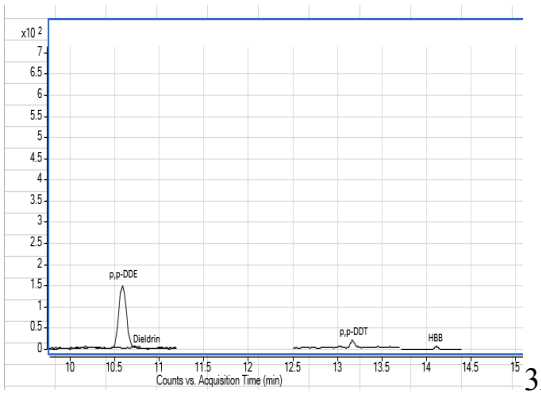
Pearson Correlation Coefficients, N = 35	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.92122
Quantitated DBSDDE P-value	<.0001
Spearman Correlation Coefficients, N = 35	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.89076
Quantitated DBSDDE P-value	<.0001
After removing 30%	
Pearson Correlation Coefficients, N = 34	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.9193
Quantitated DBSDDE P-value	<.0001
Spearman Correlation Coefficients, N = 34	
Prob > |r| under H0: Rho=0	
	SerumDDE
Quantitated DBSDDE	0.8906
Quantitated DBSDDE P-value	<.0001

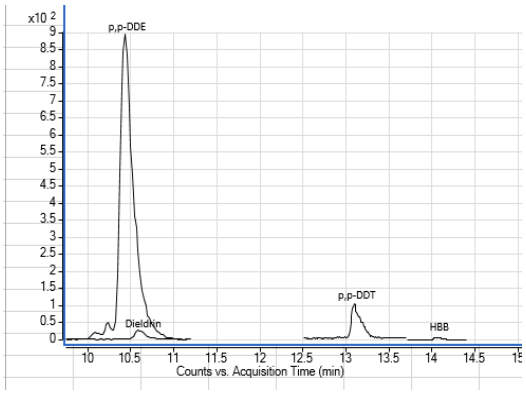
Figure 1.1

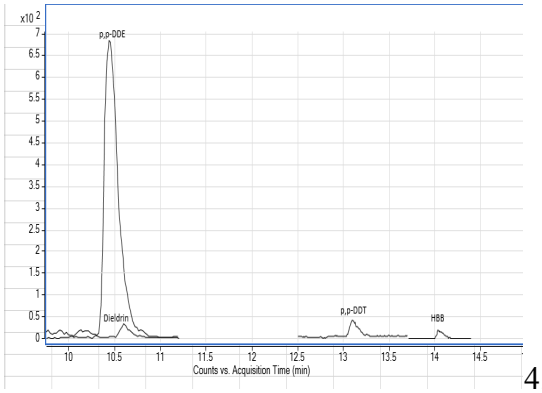
Extracted ion chromatograms depicting 1. Standard 4 – 0.05 ng/mL equivalent calibrant, 2. A self-scaled 0.05 ng/mL fortified in-house dried blood spot QCH, and B scaled to NIST 0.05 ng/mL fortified in-house dried blood spot QCH, 3. A self-scaled 0.05 ng/mL fortified in-house pooled serum QCL, and B scaled to NIST 0.05 ng/mL fortified in-house pooled serum QCL, 4. Unknown dried blood spot sample, and 5. Unknown serum sample.

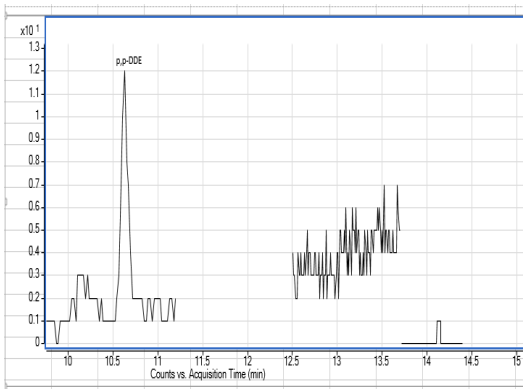
1. 

2A 

2B. 

3A. 

3B. 

4. 

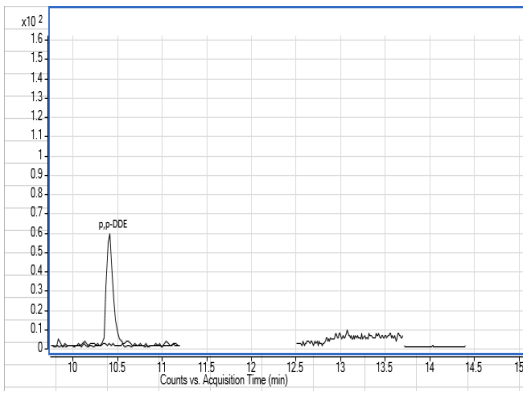
5. 

Figure 1.2

Percent agreement between serum DDE and quantitated DBS DDE concentrations.

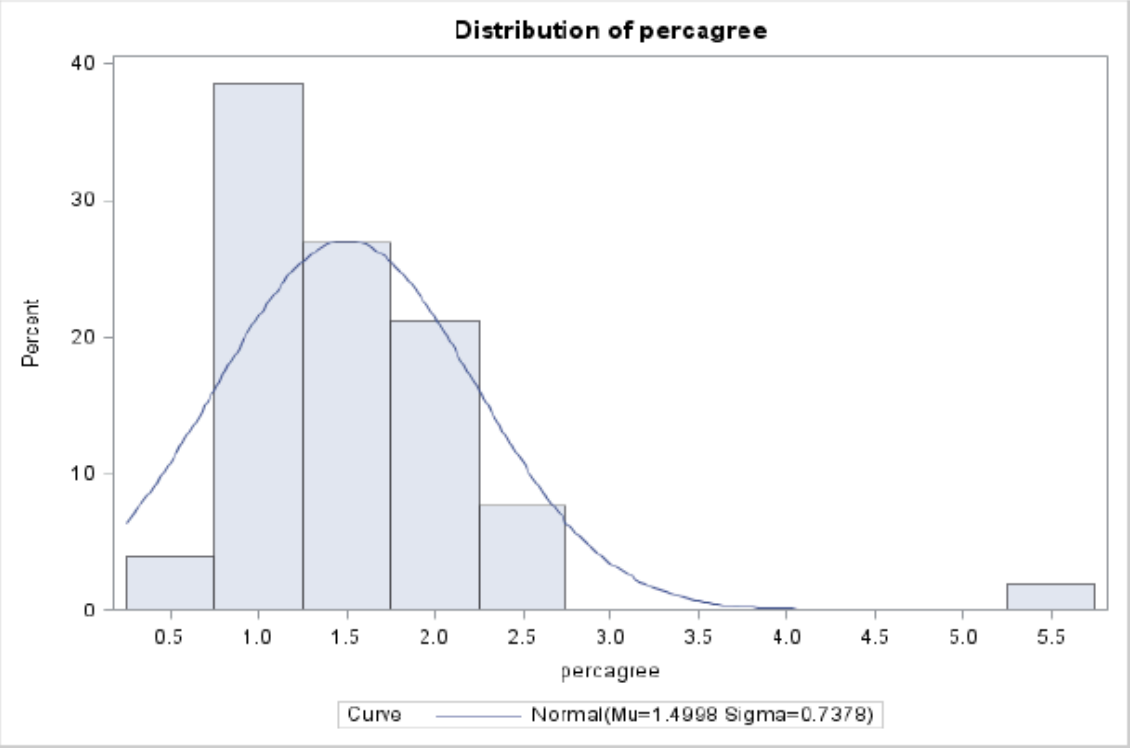
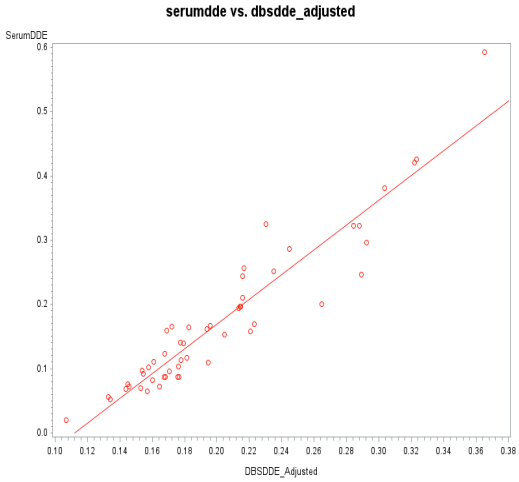


Figure 1.3

Correlations between A. serum DDE and DBS DDE quantitated, B. exposure age and serum DDE, C. exposure age and serum DDT (n=23), D. exposure age and DBS DDE quantitated, E. blood draw age and serum DDE, F. blood draw age and serum DDT (n=23), and G. blood draw age and quantitated DBS DDE.



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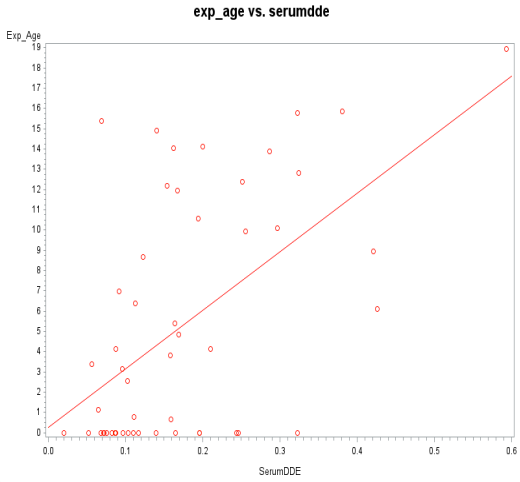

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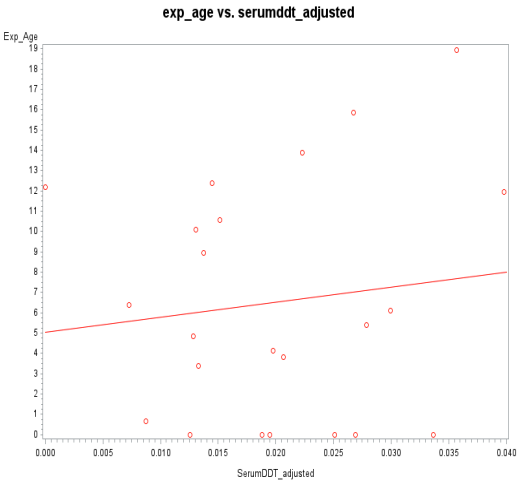

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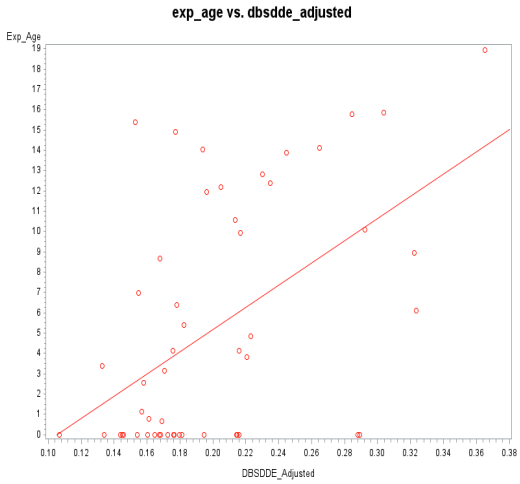
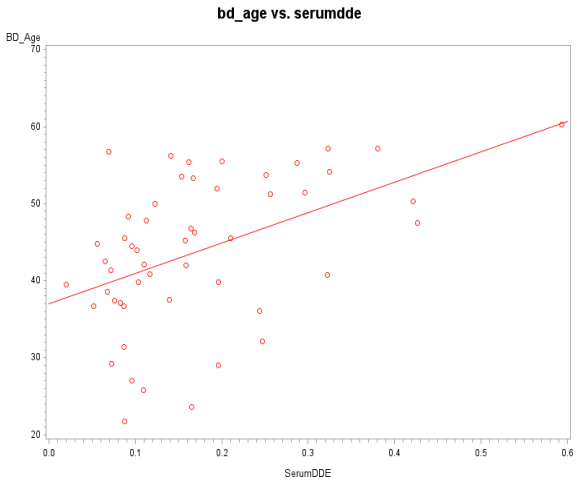


D.


bd_age vs. serumdde
BD_Age
70
60
50
40
30
20
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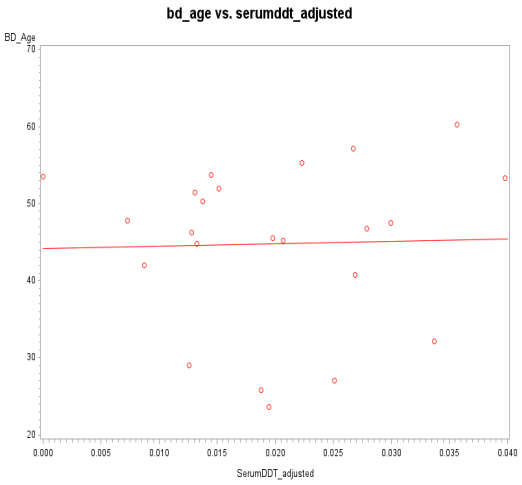
0.1
0.2
0.3
0.4
0.5
0.6
SerumDDE

E.


bd_age vs. serumddt_adjusted
BD_Age
70
60
50
40
30
20
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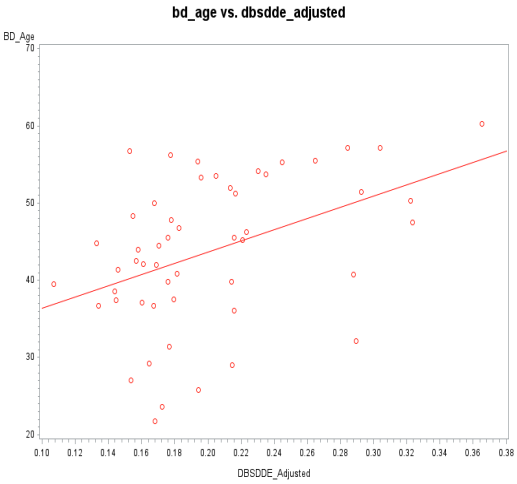
0.005
0.010
0.015
0.020
0.025
0.030
0.035
0.040
SerumDDT_adjusted

F.


bd_age vs. dbsdde_adjusted
BD_Age
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60
50
40
30
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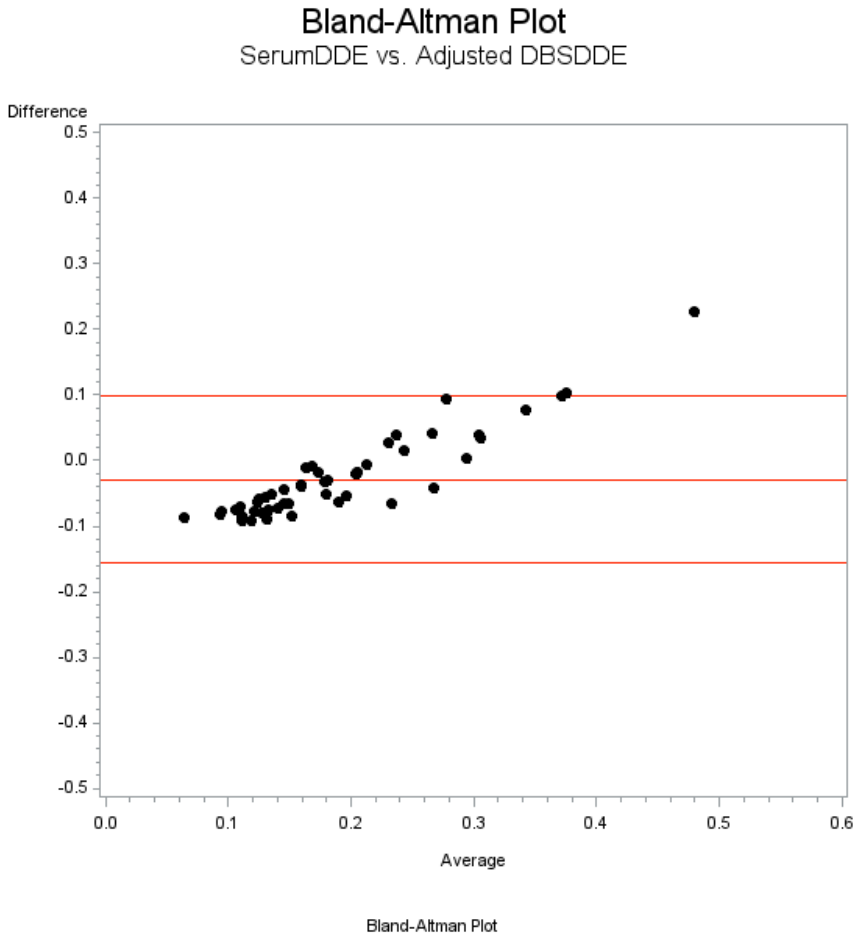
0.12
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0.28
0.30
0.32
0.34
0.36
0.38
DBSDDE_Adjusted

G.

Figure 1.4

Bland-Altman plot assessing agreement between serum DDE and quantitated DBS DDE.



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