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Performance and Storage Integrity of Dried Blood Spots for PCB, BFR and Pesticide Measurements

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Abstract

Dried blood spots (DBS) can provide accurate and valuable estimates of exposure to environmental toxicants, and the use of information derived from archived newborn DBS information has enormous potential to open up new research on the impacts of early chemical exposure on disease. Broad application of DBS for the purpose of quantitative exposure estimation requires robust and validated methods. This study investigates the suitability of DBS analyses for population studies of exposure to three chemical groups: polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs), and chlorinated pesticides. It examines background (matrix) contamination, recovery and extraction variability, sensitivity, and storage stability. DBS samples prepared using 50 µL of adult blood were analyzed by GC/MS, and method performance was confirmed by using certified materials and paired DBS-blood samples from six volunteers. Several of the target compounds and their degradation products have not been previously measured in DBS. All target compounds were detected in DBS samples collected from the volunteers. Sample DBS cards showed background contamination of several compounds. When stored at room temperature, target compounds, excluding PBDEs, were stable for up to one month. When refrigerated or frozen, stability was acceptable for all compounds up to one year, and multiyear storage appears acceptable at colder (e.g., –80 °C) temperatures. Multicompartment models may be used to estimate or correct for storage losses. Considering concentrations of contaminants for adults and children reported in the literature, and experimental values of detection limits and background contamination, DBS samples are suitable for quantifying exposures to many PCBs, BFRs and persistent pesticides.

Keywords

Blood; bloodspot; DDT; exposure; organic compounds; PBDEs; PCBs; Biomonitoring; child exposure; flame retardants; pesticides; emerging contaminants

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1. Introduction

All U.S. states have mandatory newborn screening programs for diseases (e.g., phenylketonuria) in their health preventative services that use the collection and analysis of biological specimens, most commonly newborn dried blood spots (DBSs). Fourteen states store biological specimens for at least 21 years, and eight programs report indefinite storage [1, 2]. States including Michigan and California have extensive archives that can be utilized for a wide variety of scientific purposes. For example, the Michigan Neonatal Biobank (MNB) has been collecting DBSs for every birth in Michigan since 1984, and its repository now includes millions of specimens. Such archives provide a unique and potentially powerful resource for retrospective assessment of environmental exposures during the prenatal period. The potential of DBS archives for epidemiological and other purposes has been recognized for several decades [3]. Potentially, a huge range of analytes could be examined using DBS, and the use of this information in cohort studies of well-phenotyped individuals has enormous potential to open up new research on the impacts of early chemical exposure on disease. The use of DBS samples also has several advantages as a biomarker sampling and storage technique for prospective investigations: the method is simple; less invasive than venipuncture; collection, transport and storage costs are low; and increased safety given the minimal volume of blood and reduced risk of infection [4, 5]. As a result, DBS may provide a cost-effective alternative to venipuncture.

DBS collection involves applying small volumes of blood, e.g., from a heel stick, to a sample collection card. Typically, 4 or 5 spots are made, dried at ambient temperature, and then sent to laboratory for analysis and then archived. Transport and storage conditions have changed over time. In Michigan, for example, DBS cards collected from 2009 to date are stored at -20°C , cards from 1998 through 2008 are refrigerated (controlled temperature and humidity), and older cards are stored at ambient temperature [6]. When received at the laboratory, a fixed area of the blood spot is extracted, typically using a 3.2 mm round punch. The size of the disk sampled provides the volumetric data for quantitation [7]. One limitation of the technique, however, is that the amount of blood in the punch depends on the spreading of blood, which is determined by the hematocrit concentration, which can vary for neonates over a relatively large range. [8] Multiple analytes can be determined from a single DBS using multiple punches. If a larger sample quantity is needed, e.g., due to detection limit issues, the entire DBS may be used or multiple punches may be used to form a composite sample [9].

Applications of DBS measurements for bioanalyses and pharmaceutical residues (and their derivatives) have been recently reviewed [4, 5, 7, 10–14]. The number of reports describing the methods and experience with DBS for exposure assessment purposes is comparatively sparse. Environmental applications have focused on several trace metals, e.g., lead and cadmium [15–17], and methods for the more prevalent metals, specifically lead, are considered to be reasonably well-validated [18]. However, DBS have been used for other analytes. For example, in mostly pilot studies, human and avian DBS samples were used to measure additional trace metals [15], cotinine [19], perchlorates [20], cysteinyl adducts of benzene oxide [21], bisphenol A [14, 22], and persistent organic compounds, e.g., pesticides

[23–25], PCBs [24, 26], PBDEs [9, 24, 26], and fluorinated compounds like PFOS [14, 24, 27, 28].

Broad application of DBS for quantitative exposure estimation purposes requires validation, reference materials, control samples and other quality assurance (QA) elements that meet data quality objectives [29, 30]. A number of issues in the collection, storage, processing, analysis and use of DBSs have been recognized [7, 10, 12, 13, 18], and most pertain to environmental toxicants. These include: loss of analytes by enzymatic action (or other mechanisms) during the collection, spotting and drying stages [31, 32]; differences between analyte profiles measured using DBS and plasma [33]; effects of storage times and storage conditions on sample integrity [34, 35]; the consistency of blood spot punches and chromatographic considerations on sample cards [36]; variability, recovery, sorption and other issues involved in extracting analytes from sample cards [11, 26, 37]; variability in blood volumes, irregular geometry of the DBS itself, and punch size [8, 38, 39]; background (matrix) contamination on the filter paper [15, 20]; within- and between-lot variability of the sample card properties and contamination [40]; chromatographic, carryover and other separation issues in analysis and quantitation [41]; limited sensitivity of methods, which can be particularly acute given the small volume of sample available [5, 10]; and a lack of standard reference materials (SRMs) to help ensure that measurements meet quality assurance criteria. (For example, SRMs are available from NIST for (persistent) organic contaminants in non-fortified human serum [SRM-1957] and metals in caprine (goat) blood [SRM-955c]). While progress is being made on many of these fronts, few investigators have reported basic performance criteria, including precision, accuracy, linearity, sensitivity, selectivity, and storage stability [7].

This study addresses the suitability of DBS measurements for population studies. It evaluates background (or matrix) contamination, recovery and extraction variability, sensitivity, and storage stability of persistent organic pollutants (POPs) on DBS. Three groups of POPs are tested: brominated flame-retardants (BFRs), chlorinated pesticides, and polychlorinated biphenyls (PCBs). Each group includes toxicants found in human blood. While dependent on many factors, e.g., age and diet, concentrations of the selected contaminants can be relatively high. The performance of DBS measurements and ability to meet QA goals are evaluated using both real and laboratory-prepared DBSs, reference materials, and tests over a one-year period.

1 Methods

1.1 Compound selection

Target compounds were selected considering prevalence and toxicity. We examined recent literature, including the latest National Health and Nutrition Examination Survey (NHANES) reports [42] and studies specific to newborns and children. While NHANES examines adults, it has the advantages of employing extensive and well-documented QA procedures and a large and nationally representative sample.

Four to 11 compounds in three groups of chemicals were selected. These included PCBs (congeners 28, 138, 153, 180, 105, 118, 194, 206, 209, 170, 187); persistent pesticides

(trans-nonachlor, dichlorodiphenyldichloroethylene (p,p'-DDE), β -hexachlorocyclohexane (β HCH), hexachlorobenzene (HCB), and brominated flame retardants (BFRs) including 2,2', 4,4',5,5'-hexabromobiphenyl (PBB-153), 2,2',6,6'-tetrabromo-4,4'-isopropylidene diphenol (TPBB-a), and PBDEs (congeners 28, 47, 99, 100, 153, 154). Table 1 shows the names and properties of these compounds.

1.2 Background contamination

Background (or matrix) contamination on sample cards used to collect DBS was evaluated using two types of new cards used by the MNB and many other states (CF10, CF12, W-903, Whatman, Sigma Aldrich, St. Louis, MO; and A-226, Ahlstrom, Alpharetta, GA). Circular punches (15 mm dia) were taken from empty areas (without blood) of the card. To increase sensitivity, representativeness and efficiency, composite samples were used, each consisting of two punches from a single card. Ten cards of each type were tested, each from a single lot.

1.3 Volunteer tests

Measurements of POPs in DBS and whole blood were compared among samples collected from six adult volunteers (aged 20 to 64 years) from different countries. Blood was collected in 7 mL tubes (Pink Top, Becton, Dickinson and Co., Research Triangle Park, NC). DBSs were prepared by placing a 50 μ L drop of whole blood using a 100 μ L syringe on unused cards (Whatman-903), which were dried overnight in a desiccator. Triplicate DBS and duplicate whole blood samples for each individual were analyzed using procedures described below. All protocols were approved by our institutional review board.

1.4 Preparation of storage stability and QC samples

DBSs for quality control (QC) and sample storage tests were prepared by aliquoting fresh blood (composite from volunteers) treated with EDTA. The sample was mixed for 10 min, allowed to equilibrate overnight, and 50 μ L aliquots were applied to unused sample cards (Whatman-903) as described above. This blood volume was selected as typical of the quantity found in NBS. [7] Cards were dried overnight and stored at several temperatures.

A total of 125 DBSs and 125 blanks were prepared using 25 sample cards for each subsample type. (Additional cards were prepared and stored for possible future studies, e.g., further aging tests and interlaboratory comparisons.) Prepared DBS cards were individually wrapped in cleaned aluminum foil (rinsed with acetone and dichloromethane (DCM), and then baked overnight at 260 °C), and placed in groups of five in sealed polyethylene bags.

1.5 Sample stability tests

A series of tests was designed to simulate DBS transport and storage at four temperatures: 20 °C (room temperature), 4 °C (refrigerated), -20 °C (frozen), and -80 °C (ultralow freeze). The first three conditions correspond to those used in the MNB. Tests were run at nine time points (0, 1, 3, 10, 30, 100, 190, 251 and 365 days measured after sample preparation). Three replicates were used for each time point and temperature, along with QC samples, blanks and calibration checks.

1.6 Sample preparation and analysis

Sample analyses followed procedures described as NIST method 1. [43] In brief, DBS punches (15 mm dia) containing the DBS were placed in a 15 mL glass tube (Fisher Scientific, Pittsburgh, PA), and treated with hydrochloric acid (1 mL, 6 M) and then 1:1 ethanol/isopropanol mixture (6 mL). The sample was extracted twice using 1:1 hexane/methyl-t-butyl ether (6 mL, MTBE). After rotating for 2 min, the organic layer was transferred to a new tube with 1% potassium chloride (4 mL) and re-extracted with 1:1 hexane/MTBE (3 mL). The organic phases were combined, and the solvent volume was reduced to 1 mL by evaporation under a gentle N₂ flow. Next, the concentrated sample was eluted through a sulfuric acid/silica gel column (6.6 g silica, 3.3 g sulfuric acid at the top; 0.1 g silica, 1 g sulfuric acid at the bottom). Prior to use, the silica gel was baked overnight at 280 °C and rinsed with 1:1 hexane/DCM mix (9 mL). Analytes were eluted from the column with 1:1 hexane/DCM mix (8 mL), and then evaporated under N₂ to a volume of 0.5 mL. The final extract was prepared with the addition of 0.5 mL n-nonane and evaporated under N₂ to a volume of 250 µL.

Analytes were separated and quantified using a gas chromatograph and mass spectrometer (GC/MS; 5890/5963, Agilent Industries, Palo Alto, CA, USA) equipped with a DB-5 column (30 m length, 0.25 mm id, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). Each run used a 2 µL injection, 10 µL injector syringe, injector temperature of 280 °C, helium as the GC carrier gas (flow rate of 0.7 mL/min, pressure of 5.43 psi, and average velocity of 31 cm/s), and required from 34.5 (PBDEs) to 69.5 min (pesticides).

GC temperature programs and MS settings were optimized for each compound group. Five temperature programs were used. For PCBs, the initial oven temperature setting was 80 °C, which was held for 1 min, then ramped at 20 °C/min to 150 °C, then ramped at 2 °C/min to 250 °C, held for 4 min, ramped at 30 °C/min to 300 °C, and finally held for 6 min (total run time of 66.17 min). For pesticides other than toxaphene, the initial temperature was 80 °C, held for 1 min, ramped at 20 °C/min to 150 °C, ramped at 2 °C/min to 250 °C, held for 4 min, ramped at 10 °C/min to 300 °C, and then held for 6 min (total of 69.5 min). For toxaphene, the initial temperature was 80 °C, held for 1 min, increased at 10 °C/min to 200 °C, increased at 1.5 °C/min to 230 °C, increased at 10 °C/min to 300 °C, and held for 6 min (total of 46 min). For tetra- through octa-homologues of PBDEs, temperatures started at 80 °C, held for 2 min, and then ramped at 10 °C/min to 300 °C (total of 34 min). For nona-through deca-homologues of PBDEs, the temperature started at 80 °C, held for 2 min, increased at 50 °C/min to 300 °C, and then was held for 55 min (total of 61.4 min). The MS detector was operated in negative chemical ionization selective ion monitoring (NCI SIM) mode using methane as the reagent gas. Ions and retention times are listed in Supplemental Table 1.

Analytes designated as total were determined as the sum of individually quantified constituents calibrated against authentic standards, e.g., Σ_{11} PCB is the sum of the 11 congeners noted above, and Σ_6 PBDE is the sum of 6 congeners. In this application, measurements below the detection limit in sums were set to zero; this made no practical difference since concentrations of most samples considerably exceeded detection limits, and only data for the most abundant compounds are presented in this paper.

1.7 Calibration and quality assurance (QA)

Calibration standards were prepared using certified calibration standards (CDC persistent pesticides (PPC), PCB and PBDE calibration solutions, Cambridge Isotope Laboratories) at concentrations of 0.01, 0.1, 0.5, 1, 10 and 50 ng/mL. This range was selected to span the range expected in the adult samples.

Table 1 lists instrumental detection limits (IDLs) for the target compounds, defined as the amount of analyte for which the signal-to-noise ratio of the peak is equal to three, based on analyses of low concentration samples. The IDLs assume analyses using an extraction volume of 250 μ L. They are appropriate for the sample integrity and other analyses in this paper. Because they may not include all sources of error, they are defined as IDLs, and not as method detection limits.

A minimum of three replicates were analyzed for each sample type and time point. Internal standards (IS), quantified in each run, consisted of 200 μ L of labeled PCB, pesticide and PBDE mixtures (EO-5275, EC-4060, ES-5478 CIL, Tewksbury, MA), and 15 μ L of the labeled internal standard, which were injected into each GC vial just before the GC/MS run. Labeled PCB standards were used as internal standards for the PBDEs. Each set of analyses used quality control (QC) samples processed in parallel with samples, and QC analyses included: drift checks (using repeated analysis of a standard injected every fifth sample), blanks, linearity, spike recovery, and SRM checks. Results were accepted only when duplicate values varied by less than 10%, linearity defined using a R^2 exceeded 0.999; and measurements of SRMs were within 20% of certified values (see below). All spike recoveries were in the range of 78 to 104%. Blank contamination in standards and blood collection materials was negligible (<detection limit), although (background) contamination was found on unused cards, as discussed later.

The accuracy of both DBS and blood measurements were determined using a standard reference material (SRM 1957, Organic Contaminants in Non-Fortified Human Serum, NIST, Gaithersburg MD USA), which was reconstituted by adding 10.7 mL of HPLC-grade water, sonicating for 15 min, and equilibrating overnight under refrigeration. Then, reconstituted serum was analyzed as described in Section 1.6. The accuracy of DBS measurements was evaluated by preparing paper cards with 50 μ L of reconstituted serum (applied in stages so that the DBS did not exceed a diameter of 15 mm). Cards were then dried, extracted and analyzed. With several exceptions, these measurements ranged from 81 to 119% of certified values for blood, and from 98 to 115% for DBS. Measurements were slightly elevated (127 to 139% of certified values) for PCB-105 and PCB-194 in the DBS samples, and for HCB in blood (127%), which may have resulted from imperfect peak separation. Measurement variability was 15%, below the 20% limit. Overall, the blood and serum measurements closely matched certified values, demonstrating excellent performance of extraction and analysis steps. (Section 2.6 of this paper, Strengths and Limitations, discusses several additional design and methodological issues that might affect QA and study results.)

1.8 Data analysis

Analyte stability was assessed by comparing concentrations after various storage periods to concentrations obtained in the initial determination, measuring what has been termed “incurred” sample stability (as distinguished by comparison to the nominal values of QC samples, which is called “QC” sample stability) [5]. Using data from the storage tests, loss rates per year were estimated using linear regression models and multi-compartment exponential models, which represent fast and slow loss processes separately, and which can provide excellent explanatory ability [44–46]. Model parameters were estimated by minimizing sum of squared residuals.

2 Results and Discussion

2.1 Background contamination

Trace levels of several target POPs were found on unused cards (Table 2). PCB-170+190, 105 and 194 were found at blood concentrations equivalent to 5 ± 2 , 8 ± 1 and 19 ± 3 ng/L, respectively, and at higher levels (35 ± 3 , 17 ± 2 and 24 ± 3 ng/L) in the Ahlstrom cards (mean and standard deviation). BDE-47 was found in both cards at similar levels (35 ± 3 and 33 ± 3 ng/L for Whatman and Ahlstrom cards, respectively.) Background contamination was quite consistent across the card samples (typical coefficient of variation around 10%), all of which were drawn from the same lot. These contaminants were confirmed to be in the blanks, and not in materials used for extraction and sample processing. No additional contamination was found after storing samples for 1 year, with the exception of PCB-209 found in the final months (as discussed later in the sample storage tests). In the storage tests, the background contamination was consistent (coefficients of variation from 6 to 10%, $n=96$), indicating that the contamination source was the original card, and that storage did not add further contamination.

It is not surprising to find trace levels of PCBs and PBDEs on unused cards since these chemicals have been manufactured in large quantities, have been used indoors, and are persistent. Contamination can arise throughout the card's lifecycle, e.g., manufacturing, packaging, handling, labeling, transportation, processing, and storage. The sources, types and concentrations of contaminants may vary over time, e.g., an analysis of cards manufactured from 1987 to 2009 showed significant declines of BDE-47, PCB-153 and PCB-194 concentrations, but constant levels of other PBDEs and PCBs [26]. This study reports levels of BDE-47 and PCB-194 on unused cards from 1987 (7,000 and 11,000 ng/L, respectively) that are two to three orders of magnitude higher than measured in the present study. Since lot-to-lot variation is likely, sampling and analysis of a punch taken close to the archived DBS is recommended, using areas at least 1 mm from the edge of the blood spot [5, 26]. If background contamination is consistent across the card, and small relative to levels expected in the population, then background corrections can be used to avoid overestimating concentrations. Such corrections may be important for some PCB and BDE congeners.

The background contamination in blank cards is compared to contaminant levels expected in the population as estimated using NHANES, which is probably the best population-oriented study for the U.S. population although POPs were measured in only adults. As shown in

Table 2, background levels of PCBs-170+190, 105 and 194 approach or exceed median blood levels, while the BDE-47 background level represents 28% of the median level. Thus, uncorrected DBS measurements may have limited utility for these three PCBs, although more exposed individuals (above 75th to 90th percentiles) are easily identified. These results do not reflect card-to-card or lot-to-lot variation in background contamination levels.

Analysis of smaller punches within the DBS, as discussed below, may lower levels of background contamination. This is possible since the perimeter area of the 15 mm dia punch used to sample the roughly 10 mm dia DBS included card material with little or no blood. However, differences are not expected to be large.

2.2 Volunteer tests

Figure 1 contrasts levels of PCBs, pesticides and PBDEs measured on DBS across six volunteers. All target compounds were detected. This small sample showed considerable variation. For Σ_{11} PCB, factor of two differences were seen, and the Russian and Indian males had the highest levels (3,800 – 4,200 ng/L), well above US norms (shown later in Table 2), while the US male and female had the lowest levels (1,900 – 2,500 ng/L). Pesticides showed larger differences: the two Chinese females had very high levels of p,p'-DDE (84,000 – 104,000 ng/L); the US male and female had the lowest levels (300 – 500 ng/L). Concentrations of nonachlor, HCH and HCB were much lower but followed similar trends. Again, the variation and range are high compared to US norms. Σ_6 BDE levels ranged from 1,600 ng/L (Russian male with tri-, tetra, penta- and hexa-congeners detected) to only 50 ng/L (US female, only BDE-47 was detected near the background level). For the first time, we report levels of PBB-153 and TBBP-a in DBS (120 and 940 ng/L, respectively), which were found in one individual (64 year-old Russian male); these compounds were not detected in other volunteers (all below 40 years of age). PBB is an older flame retardant that was phased out in the 1970s following a contamination event in Michigan; TBBP-a remains widely used in circuit boards and other applications [42].

DBS and whole blood concentrations showed high correlation (Deming regression correlation = 0.80), however, DBS measurements were biased downwards by an average of $11 \pm 9\%$ (n=124) from whole blood measurements (see Bland-Altman plot, Supplemental Figure 1). While most measurements were within 20% and are thus deemed acceptable, the relative differences tended to increase at low (<250 ng/L) concentrations, a trend suggestive of detection limit issues and imperfect integration of smaller peaks. Other possible biases, e.g., incomplete extraction from the card, or chromatographic drift beyond the 15 mm punch, appeared minimal. However, further evaluation using larger samples is suggested since our samples came from only six individuals. Such analyses might evaluate the performance of different analytical procedures for specific compounds or classes of compounds, as well as effects associated with variation in hematocrit levels. [8, 47]

These results demonstrate the value of DBS for assessing current exposures. The results demonstrated large differences in exposure in a small set of individuals. While most uses of the target POPs are now restricted, these chemicals have been applied widely in household, agricultural and other applications. Exposures can be attributed to the large reservoirs of “legacy” contaminants (e.g., DDT and PCBs) remaining in soils and sediments, the large

quantities of PBDEs and other flame retardants in building materials and furnishings that accumulate in dust and elsewhere, the slow breakdown of these compounds, and the tendency to accumulate in the food chain [48–51]. The use of DBS may be especially valuable in estimating exposures among individuals potentially exposed from about 1960 to 2005 when many POPs were heavily applied, and for countries where persistent pesticides are still used.

2.3 Storage stability

Trends of concentrations in DBS samples stored at –80, –18, 4 and 20 °C for one year are shown in Figure 2. At –80 and –20 °C, concentrations of each POP group showed flat or only very slowly declining trends, indicating high sample integrity. In these samples, losses were minimal for most of the target PCBs and persistent pesticides. Several compounds in these groups showed small losses, e.g., 4 to 9% loss per year for PCB-28+31, PCB-187+182 and HCB. Linearized loss rates across the compound groups at each temperature are listed in Table 3, and loss rates for individual compounds are in Supplemental Table 2. All PBDEs (except BDEs-99 and 100) showed faster losses, 9±7% loss per year at –18 °C and 8±5% at –80 °C across the six congeners (average and standard deviation). At 4 °C, loss rates increased to an average of 17±17% for the PBDEs. Again, BDEs-99 and 100 had the lowest loss (4–5% per year). Pesticides and PCB losses remained below 5% per year (on average). Loss rates increased to 12±7% per year for the PCBs, 9±6% for the pesticides, and 23±15% for the PBDEs. Loss rates of compounds within a POP group tended to increase with the compound's volatility, lower molecular weight, and lower octanol-water partition coefficients (K_{ow}), e.g., $r=0.61$ between loss rates and $\log K_{ow}$ for the 11 PCBs stored at –18 °C. At both –4 and 20 °C, the chromatograms showed peaks believed to include hydroxy-BDE, which suggests PBDE breakdown products. At –20 °C and –80 °C, no such peaks were observed, and the abundance of the target compounds remained unchanged.

The laboratory stability tests indicate that many POPs can be measured with minimal or acceptable error (<20%) in refrigerated or frozen DBS samples that have been stored for a year and potentially much longer periods. However, this does not apply to PBDEs for DBS samples stored at room temperature. These tests also show smooth and gradual changes, indicating that a correction factor might be used to compensate for these losses. For many target compounds, losses remained small after one year and curvature in concentration trends (Figure 2) could not be discerned. For storage at room temperature, however, exponential-type models appear appropriate. A two compartment exponential model, representing fast and slow loss processes, was applied:

$$C_t = C_0 [\alpha \exp(-\beta_1 t) + (1 - \alpha) \exp(-\beta_2 t)] \quad (1)$$

This model was estimated for the average change in the six PBDEs at 20 °C. In this case: C_t and C_0 = BDE concentration measured at time t (days); fitted weight $\alpha = 0.8325$; and fitted loss rate coefficients β_1 and $\beta_2 = 0.000118$ and 0.0161 days^{-1} , respectively. The fast process (represented by β_2) accounted for the initial loss (about 17%) with a half-life of 0.12 years; the slow process accounted for the remaining loss with a half-life of 16 years. Model fit was excellent ($R^2 = 0.97$). Eq. (1) predicts that across the six BDE congeners, the concentration on a DBS stored at 20 °C for 2 years will be 76% of the initial concentration, and 54% after

10 years. For the 11 PCBs at 20 °C, model parameters were $\alpha = 0.870$; $\beta_1 = 0.00009232 \text{ days}^{-1}$, $\beta_2 = 0.00285 \text{ days}^{-1}$, and $R^2 = 0.99$; and the fast process accounted for 13% of the loss with a half-life of 0.67 years; the slow process had a half-life of 21 years; and storage for 2 and 10 years would retain 83 and 62% of the initial concentration, respectively. For the four pesticides at 20 °C, $\alpha = 0.9545$, $\beta_1 = 0.00008629 \text{ days}^{-1}$, $\beta_2 = 0.01832 \text{ days}^{-1}$, and $R^2 = 0.97$; the fast process accounted for 5% of the loss with a half-life of 0.10 years; the slow process had a half-life of 22 years; and storage for 2 and 10 years would retain 90 and 70% of the initial concentration, respectively. The linear and exponential models might be combined to represent multiple phases of storage, e.g., PBDE analyses on DBS stored at room temperature for 2 years and then refrigerated for 2 years might be anticipated to have 68% of the initial sample remaining (83% using the exponential model multiplied by $100 - (2 \times 9.1\% \text{ per year})$ from the linear model, see Table 3). While demonstrating the use of such models, such extrapolations can involve considerable uncertainty, especially because our data extends to only one year of storage and a single test concentration was used.

Information on DBS storage integrity as a function of storage time and temperature is limited [5, 7]. Many analytes are considered stable in DBS stored at ambient temperatures for several days, long enough for samples to be sent through the ordinary mail. A recent report indicates that 14 PCB and 5 PBDE congeners were stable for 30 days (within $\pm 15\%$ of values measured on day 0) when stored at room temperature [26]. No other literature regarding longer term storage was located. Other data gaps include stability at elevated temperatures, which may be encountered during transport [7], and the effect of repeated freeze/thaw cycles. The present study was limited to storage at a constant temperature, but this remains a key requirement for DBS used to estimate retrospective exposures.

2.4 Suitability determination

The suitability of DBS for measuring specific contaminants depends on the combined effects of method sensitivity, storage stability, background contamination, extraction/analysis variability and other factors, all relative to the levels and variation of contaminant in newborns or other populations of interest. To identify analytes for which DBS analyses may provide useful information, DBS measurement performance is compared to contaminant levels expected in the population. Table 2 lists concentrations reported in NHANES for target compounds. (In NHANES, nonachlor was not measured; PBDE were reported using lipid-adjusted concentrations which were converted to ng/L blood using the median ratio of lipid-adjusted to serum concentrations of 152.4, which was very consistent across all POPs; concentrations were reported as ng/g, and the minor density difference between serum and water was ignored.) NHANES levels are compared to the limit of quantitation (LOQ), estimated using a signal/noise ratio of 10 (3.33 times the IDL shown earlier in Table 1). In all cases, blank concentrations fell below LOQs (although blank levels exceeded IDLs for PCBs 118, 105 and 194 and BDE-47). Table 2 lists ratios of NHANES levels to LOQs. Higher ratios are desirable and mean that the compound can be reliably detected. Table 2 also provides a summary rating of the suitability of DBS measurements, where “excellent,” “good” and “fair” respectively indicate that the median, 90th or 95th percentile concentration exceeded the LOQ, and “poor” indicates that all three concentrations fell below the LOQ. Thus, an “excellent” rating suggests that the analyte can be measured reliably in most

samples; “fair” means it will be detected reliably in only samples with the highest concentrations. Such ratings could use other criteria as well incorporate storage integrity and other factors that are relevant to the study design and purpose.

These ratios and the overall suitability ratings in Table 2 show that DBS can be used for most of the PCBs (except PCB-28); PCBs 105, 180 and 194 may need background corrections. For pesticides p,p'-DDE and β -HCH, DBS samples will provide excellent and good performance, respectively; HCB may be measurable in at least a fraction of the population. For the BFRs, BDE-47 and 153 can be quantified, as can higher concentrations of BDE-100 and 153. These results pertain to the DBS extraction and analysis protocols described in the methods and blank levels in new cards.

The US NHANES measured about 2,000 individuals aged 14 years and older in 2003–4 using a population-based sampling strategy, and is one of the largest biomarkers studies. While NHANES may not be an entirely relevant comparison for newborns, the range of POP concentrations in NHANES approximately matched (factor of two) PCB, BFR and pesticide levels reported in several recent studies [52, 53]. Notably, pesticide exposures in some countries can considerably exceed levels found in the USA (as suggested by results for the six volunteers discussed earlier). With these caveats, the NHANES data have relevance for the evaluation of the suitability of DBS measurements.

2.5 Improving sensitivity

For most target compounds, detection limits and not the background contamination is the limiting factor. Our GC/MS method used 2 μ L injections from sample extracts reduced to 250 μ L, representing analysis of a 0.4 μ L blood sample (assuming a 50 μ L, 10 mm dia blood spot). While the extraction volume could be further reduced to improve detection limits, e.g., available autosamplers can handle volumes down to 10 μ L using conical tube inserts, this may adversely affect precision and limit opportunities for replicate and other analyses. Alternative methods, such as microextraction by packed sorbent or other means [54], can provide analysis of essentially the entire extracted sample, providing up to a 125-fold increase in mass to the detector with the simplifying assumption that sensitivity, selectivity, noise, and other factors affecting detection limits are comparable. While these assumptions are not completely realistic and they do not account for replicates, such methods might substantially increase sensitivity, as well as greatly simplify sample cleanup and preparation. In addition, the use of new instrumentation, such as magnetic sector mass spectrometers, can attain much lower detection limits and might be applicable for DBS analyses.

More sensitive methods also might permit the use of only a subsample of the DBS, which is particularly important for biorepositories unwilling to surrender the entire DBS. For example, a 3.2 mm dia punch taken from the DBS is commonly used for bioanalyses of newborn DBS. Assuming that a DBS from a typical 50 μ L blood sample forms a homogeneous 10 mm dia spot (79 mm²), then a 3.2 mm dia punch will yield 10% of the area (8 mm²) and a sample equivalent to 5 μ L blood. If the entire subsample can be utilized in a single analysis (e.g., using on-line concentration), then the 3.2 mm punch would provide up to a 12.5-fold increase in sensitivity compared to the method used in this paper assuming

that other parameters remain unchanged. Further research is needed to address sensitivity issues.

2.6 Strengths and limitations

This paper used well-controlled and reproducible laboratory tests to measure storage stability, background contamination, and other factors affecting the suitability of DBS measurements for a wide range of organic compounds. In practice, laboratory conditions may not represent conditions used to transport and store DBSs, in particular, changing temperature and humidity, the possibility of contamination, and other factors that might affect sample integrity. Other limitations of the work include: the small sample size used to compare measurements in blood and DBS; a potential accuracy problem noted at lower concentrations; the use of adult (not newborn) blood to develop and evaluate the method (newborn blood will have lower levels of chemicals that do not cross the placental barrier as well as more variable hematocrit levels); and the need to extend tests of sample storage to multiple years, thus enabling evaluation of the integrity of DBS samples archived for a decade or more. However, potential issues related to accuracy or hematocrit seem unlikely to alter study results since entire adult blood spots were used and analyses focused on relative concentrations, e.g., ratios of measurements in blood levels to detection limits or background concentrations, and since temporal changes in the sample storage/integrity tests were expressed relative to initial measurements.

3 Conclusions

The GC/MS method developed to measure PCBs, PBDEs, PBBs and persistent pesticides using DBS performed well, as confirmed using certified SRMs and other analyses. All target compounds were detected in blood samples and DBS collected from six volunteers, and the two sample types showed good agreement. Cards used for DBS samples had background contamination of several target POPs, but samples were not further contaminated and sample integrity remained high in storage extending up to one year. Storage stability of target compounds excluding PBDEs was acceptable for DBS stored at room temperature for up to one month; refrigeration or freezing is needed for longer periods. Multiyear storage appears acceptable if samples are stored at -80°C . A multicompartment model is presented that may be used to estimate (and possibly correct for) storage losses. Several analytes not previously measured in DBS were detected, including PBB-153, TBBP-a, and hydroxy-PBDE derivatives. Compounds potentially suitable for DBS measurements were identified by comparing experimental results to literature reports on POP exposures.

Epidemiology and exposure studies would benefit from biomarker measurements that are sufficiently accurate and precise to reduce the likelihood of exposure misclassification and bias. The use of DBS samples can be highly advantageous given their convenience, safety and low cost of sample collection and transport. DBS samples may be appropriate for certain persistent contaminants, as has been reported in a small number of studies. However, widespread application of DBS for the purpose of quantifying exposures requires an improved understanding of QA elements and methods that meet QA goals. This work addressed several research needs for DBS, specifically sample storage, background

contamination, recovery and extraction variability, sensitivity and precision, information needed to obtain accurate measurements of toxicants using DBS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DBS were used to measure PCBs, brominated flame retardants, and chlorinated pesticides
- Sample DBS cards showed background contamination of several compounds.
- Pesticides and PCBs, but not PBDEs were stable in DBS stored at room temperature.
- When frozen, multiyear storage of DBS for many organic compounds is acceptable.
- Multicompartment models may be used to estimate or correct for storage losses.
- Archives of DBSs are kept by many states and represent a valuable exposure resource.
- DBS sampling is simpler, more cost-effective, and safer than venipuncture.

Figure 1. Summary results for PCBs, pesticides and PBDEs using DBS prepared from 6 volunteers. Nationality and gender of volunteer indicated in legend.

Figure 2.

Trends of PCB, PBDE and pesticide concentrations measured in DBS and stored at four temperatures over one year. Relative concentration is referenced to the concentration measured on the first day. Average loss among compounds in each target group is shown.

Identification of target compounds, chemical properties from EpiSuite (<http://www.epa.gov/oppt/exposure/pubs/episuite.html>), instrumental detection limits (DL), and comparison to NIST standard reference material (SRM) to reconstituted serum and DBS measurements.

Table 1

Group	Chemical	Chemical name	CAS No.	Formula	Properties from EpiSuite			Detection Limit (ng/L)	Certified SRM		
					Mole. Wt.	Log Kow (oct-water)	Vapor Pres (mm Hg)		NIST (1) (ng/kg)	Serum (2) (ng/kg)	DBS (3) (ng/kg)
PCBs	PCB-28	2,4,4'-trichlorobiphenyl	7012-37-5	C12H7Cl3	257.6	5.62	4.00E-04	30	8.6	10.4	<DL
	PCB-138	2,2',3,4,4',5'-hexachlorobiphenyl	35065-28-2	C12H4Cl6	360.9	7.44	5.80E-07	10	36.9	42.2	38.3
	PCB-153	2,2',4,4',5,5'-hexachlorobiphenyl	35065-27-1	C12H4Cl6	360.9	7.75	3.43E-06	10	58.2	67.1	65.3
	PCB-180	2,2',3,4,4',5,5'-heptachlorobiphenyl	35065-29-3	C12H3Cl7	395.3	8.27	9.77E-07	17	54.5	61.3	62.5
	PCB-105	2,3,3',4,4'-pentachlorobiphenyl	32598-14-4	C12H5Cl5	326.4	6.79	6.53E-06	5	4.1	5.2	5.7
	PCB-118	2,3',4,4',5-pentachlorobiphenyl	31508-00-6	C12H5Cl5	326.4	7.12	8.97E-06	30	18.9	17.3	<DL
	PCB-194	2,2',3,3',4,4',5,5'-octachlorobiphenyl	35694-08-7	C12H2Cl8	429.8	8.68	2.87E-08	12	11.9	14.3	15.3
	PCB-206	2,2',3,3',4,4',5,5',6-nonachlorobiphenyl	40186-72-9	C12H1Cl9	464.2	9.14	7.60E-09	25	7.0	8.3	<DL
	PCB-209	decachlorobiphenyl	2051-24-3	C12Cl10	498.7	8.27	1.06E-07	50		4.3	<DL
BFRs-PBDEs	PCB-170	2,2',3,3',4,4',5-heptachlorobiphenyl	35065-30-6	C12H3Cl7	395.3	8.27	6.28E-07	30	16.2	17.4	<DL
	PCB-187	2,2',3,4',5,5',6-heptachlorobiphenyl	52663-68-0	C12H3Cl7	395.3	8.27	1.30E-07	30	15.5	16.3	<DL
	BDE-28	2,4,4'-tribromodiphenyl ether	41318-75-6	C12H7Br3O	406.9	5.88	6.70E-07	30	20.0	17.5	<DL
	BDE-47	2,2',4,4'-tetrabromodiphenyl ether	5436-43-1	C12H6Br4O	485.8	6.77	7.00E-08	30	268.0	277.4	278.3
	BDE-99	2,2',4,4',5-pentabromodiphenyl ether	60348-60-9	C12H5Br5O	564.7	6.84	3.10E-08	30	76.0	81.3	78.2
	BDE-100	2,2',4,4',6-pentabromodiphenyl ether	189084-64-8	C12H5Br5O	564.7	7.66	2.44E-08	30	49.7	58.2	53.2
	BDE-153	2,2',4,4',5,5'-hexabromodiphenylether	68631-49-2	C12H4Br6O	643.6	8.55	2.87E-09	15	61.0	67.2	64.7
	BDE-154	2,2',4,4',5,6'-hexabromodiphenylether	207122-15-4	C12H4Br6O	643.6	8.55	2.87E-09	15	7.0	6.1	<DL
	PBB-153	2,2',4,4',5,5'-hexabromobiphenyl	59080-40-9	C12H4Br6	627.6	9.10	3.32E-07	15	15.8	16.5	15.4
Pesticides	TBBP-a	2,2',6,6'-tetrabromo-4,4'-isopropylidene diphenol	79-94-7	C15H12Br4O2	543.9	7.20	3.46E-11	12	120.0	113.8	122.3
	trans-Nonachlor		039765-80-5	C10H5Cl9	444.2	6.35	2.16E-06	50	58.3	55.6	53.9
	p,p'-DDE	Dichlorodiphenyl dichloroethylene	72-55-9	C14H8Cl4	318.0	6.51	6.00E-06	90	921.0	893.2	926.1
	P-HCH	(β) hexachlorocyclohexane	319-85-7	C6H6Cl6	290.8	4.14	3.52E-05	60	31.3	36.6	<DL
	HCB	hexachlorobenzene	118-74-1	C6Cl6	284.8	5.73	1.80E-05	55	29.7	38.5	<DL

- (1) Concentration reported in standard reference material (SRM).[43]
- (2) Concentration measured in 1 ml samples of reconstituted SRM.
- (3) Concentration measured in 50 µl blood spot samples using reconstituted SRM.

Table 2
Comparison of concentrations measured in NHANES to estimated limit of quantitation (LOQ) to determine suitability of DBS measurements. BG is background contamination in cards.

Compound	LOQ (ng/L)	BG Contam. (ng/L)	NHANES Concentration			Ratio to LOQ			Overall suitability for DBS measurements / BG Notes, if any
			50th (ng/kg)	90th (ng/kg)	95th (ng/kg)	50th (ratio)	90th (ratio)	95th (ratio)	
PCB-28	100	0	30	57	67	0.3	0.6	0.7	Poor
PCB-138	33	0	95	359	477	2.9	10.8	14.3	Excellent
PCB-153	33	0	135	477	624	4.1	14.3	18.7	Excellent
PCB-180	57	35	114	409	534	2.0	7.2	9.4	Excellent, BG Correction
PCB-105	17	17	7	27	43	0.4	1.6	2.5	Good, BG Correction
PCB-118	100	ND	32	143	216	0.3	1.4	2.2	Good
PCB-194	40	24	26	96	129	0.7	2.4	3.2	Good, BG Correction
PCB-206	83	ND	NA	NA	NA	NA	NA	NA	-
PCB-209	167	ND	NA	NA	NA	NA	NA	NA	-
PCB-170	100	ND	41	144	188	0.4	1.4	1.9	Good
PCB-187	100	ND	29	115	167	0.3	1.2	1.7	Good
p,p'-DDE	300	ND	1,260	7,070	12,100	4.2	23.6	40.3	Excellent
β-HCH	200	ND	NA	216	372	NA	1.1	1.9	Good
HCB	183	ND	90	157	186	0.5	0.9	1.0	Fair
PBB-153	50	ND	14	84	178	0.3	1.7	3.6	Good
TBBP-a	40	ND	NA	NA	NA	NA	NA	NA	-
BDE-28	100	ND	NA	NA	NA	NA	NA	NA	-
BDE-47	100	35	126	558	1,068	1.3	5.6	10.7	Excellent, BG Correction
BDE-99	100	ND	NA	142	277	NA	1.4	2.8	Excellent
BDE-100	100	ND	24	121	239	0.2	1.2	2.4	Good
BDE-153	50	ND	31	214	431	0.6	4.3	8.6	Good
BDE-154	50	ND	NA	14	28	NA	0.3	0.6	Poor

(1) NA: not reported in NHANES.

Table 3

Summary of linearized loss rates (percent per year) for DBS measurements storage at four temperatures. Average rate across compounds in each group. Standard deviation in parentheses.

Group	Storage Temperature			
	22 C	4 C	-18 C	-80 C
PCBs	-11.8 (6.6)	-4.5 (5.3)	-1.5 (3.5)	-0.7 (3.7)
PBDEs	-22.7 (14.8)	-17.1 (17.3)	-9.1 (7.3)	-7.9 (4.9)
Pesticides	-8.7 (5.8)	-4.6 (3.1)	-1.7 (5.7)	-2.0 (3.0)