ANALYSIS OF POPS FROM 50 µL DRIED BLOOD SPOTS

Vining B[1]*, Hart J[1], Boivin L[1], Saul D[1], Appele R[1], Nichols J[2]

[1]SGS Environmental Services, 5500 Business Dr., Wilmington, NC, USA
[2]General Electric Company, One Research Circle, CEB 154, Niskayuna, NY, USA

INTRODUCTION

Assessment of the blood levels of persistent organic pollutants (POPs) in humans has traditionally required venipuncture and collection of relatively large sample volumes (as much as ~40 mL whole blood). These large volumes are required to obtain adequate detection limits given the low concentrations of POPs typically found in human blood. Conventional sample collection procedures present a number of difficulties including: 1) reluctance on the part of subjects to participate in resampling should analytical problems occur, and 2) materials and storage issues relating to field sampling.

The collection of dried blood spots for analysis overcomes these difficulties: Firstly, each sample with its low, 50 µL, volume results in a small (~1 cm diameter) spot on the paper. Multiple samples may be collected in a very short time, avoiding the need for resampling in the case of analytical difficulties. Additionally, field sampling becomes far more viable, as all that is needed are paper cards, lances, and sterilization materials (such as alcohol soaked wipes).

Analysis of blood spots for POPs, however, presents a different set of difficulties versus analysis of ~20 mL serum. Some initial work has been performed along these lines1, but the field is still a burgeoning one. Most notably, the detection limits required for POPs analyses are much more difficult to attain when using a sample whose volume is nominally 50 µL. Additionally, the extremely low detection limits make background contamination (especially from the paper cards themselves) a potential problem that would not be observed when using larger sample volumes.

This project targeted a specific subset of congeners determined to provide a specific exposure indication. We present here the development and initial validation work on a method for the analysis of five POPs from ~50 µL dried blood spots that we used to analyze 72 samples of spiked human whole blood.

MATERIALS AND METHODS

Each blood spot was analyzed for PCBs 101, 105, and 138, PBB-153 and lindane. All five compounds were analyzed using isotope dilution with single ion monitoring gas-chromatography coupled with high-resolution mass-spectrometry (ID-SIM-GC/HRMS). Each of the five target analytes was quantified relative to its fully 13C labeled analog (hereafter referred to as an extraction standard).

Dried blood spots were received at the laboratory already cut into strips.

Each dried blood spot was spiked with a mix containing 10 pg of each of the 13C labeled extraction standards (ES). The samples were then allowed to equilibrate for one hour. A formic acid/acetone mixture was used to disrupt protein binding, after which the spots were sonicated to remove the blood from the paper. A liquid-liquid extraction of the formic acid solution was performed using dichloromethane and hexane. After concentration of the extract and addition of 13C PCB-128 as an injection (recovery) standard (JS), the extracts were ready for analysis at a final volume of 10 µL in nonane.

GC-HRMS analysis was performed using a Waters Autospec-Premier. A 30m DB-5ms GC column was used, with an initial oven temperature of 120ºC, ramped to 325ºC over a 29 minute run time. The injector temperature was set at 280ºC. The transfer line (between the GC and HRMS system) turned out to be critical to balancing the sensitivity of response of the five compounds and was optimized at 260ºC. A 4 mL GC injection liner without glass wool was used, and an injection volume of 2 µL was necessary to obtain the desired detection limits.

To ensure adequate coverage of the low range, the instrument was calibrated with eight calibration standards, as shown in Table 1.

Table 1. Concentrations of calibration standards used in the analysis of POPs from Dried Blood Spots

ANALYTE	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8
	pg/µL	pg/µL	pg/µL	pg/µL	pg/µL	pg/µL	pg/µL	pg/µL
PCB 101	0.0125	0.025	0.05	0.1	0.2	0.4	0.8	1
PCB 105	0.0125	0.025	0.05	0.1	0.2	0.4	0.8	1
PCB 138	0.0125	0.025	0.05	0.1	0.2	0.4	0.8	1
BB 153	0.03125	0.0625	0.125	0.25	0.5	1	2	2.5
Lindane	0.1	0.2	0.4	0.8	1.6	3.2	6.4	8

As Table 1 shows, the calibration extended as low as 12.5 fg/µL for the PCBs, at which we were able to obtain signal-to-noise ratios of 10:1 (25 fg on column). Optimization of dwell times for the native (unlabeled) masses monitored assisted in making this achievement possible. Further, the m/z monitoring functions were chosen maximize sensitivity. RSDs for the relative response factors obtained for each compound ranged from 5.1% to 6.5%, indicating excellent linearity over the range indicated, as well as sufficiently strong response at the low end to prevent degradation of the signal.

The method was validated through the analysis of blood spots spiked with known amounts of native analyte prior to shipment to the laboratory. These were blind QC samples; i.e. the laboratory had no prior knowledge of the concentrations. Initially, four replicate samples were sent to the laboratory along with an equal number of blank paper samples. In a second round of validation, a triplicate calibration curve in blood as blood spots) was sent to the laboratory. In addition, several types of blank paper were sent for analysis to test various paper backgrounds with and without cleaning.

RESULTS AND DISCUSSION

First Validation Experiment:
In the first validation experiment, two pairs of spiked samples were sent to the laboratory, with eachpair at the same spiked concentration. Although the spike was evident (see Figure 1), the background from the paper was sufficiently high as to make accurate determination of the spiked concentration impractical.

In particular, the PCBs can be observed at high levels (relative to the samples) in the paper blanks. The method blank (which consisted of only solvent without paper) showed that the laboratory contributed very little to this paper blank background. Only PBB-153 was at a low enough level in the paper blanks and a high enough level in the samples to be accurately quantified.

As a consequence, before the second set of validation samples were sent, the paper cards were cleaned using a carbon dioxide regimen.

Another observation from this first study was that the sonication step had to be carefully controlled. The original extraction protocol did not call for the use of a sufficiently large volume of the formic acid solution to cover the blood spot. As a consequence, the labeled standards' recoveries for most of the compounds was poor, at ~20% or less; onlylindane was recovered well.

Second Validation Experiment:
The second set of validation samples consisted of a triplicate calibration curve (again sent blind to the laboratory) as well as a large number of blank samples to evaluate different kinds of paper and the efficacy of the carbon dioxide cleaning.

The results from these samples were mixed. Mean labeled standard recoveries ranged from 51% (lindane) to 100% (PBB 153). Recoveries were generally quite consistent, with the exception of lindane, which sometimes failed to recover at all. It remains unclear why the extraction was inconsistent in performance. Possibilities include too long or too short a sonication period, inadequate protein binding disruption, and incorrect extraction solvent.

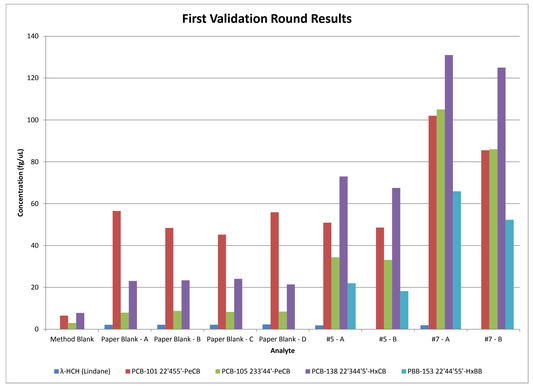

Figure 1. Results of the first round of validation testing. The method blank consisted of just solvent without paper. Although it is clear that the PCBs and PBB were spiked into the samples, the background in the paper blanks is too large to allow for reliable quantitation of the target analytes, with the possible exception of the PBB.

The results showed that less of the target analytes were recovered than expected (Figure 2), and, in some cases, showed a non-linear response. The reason for this non-linearity is not immediately evident. PBB 153, however, shows linear response with concentration, although still less target analyte was recovered than expected.

We are currently investigating the source of this discrepancy to determine if it is due to error in the preparation of the matrix-matched samples. The special nature of isotope dilution should prevent the emergence of such artifacts, provided spiking was performed correctly. Thus, if the preparation of the blood spots was correct, we would have to conclude that the spike did not fully integrate with the sample. It might be necessary, for example, to spike the blood spot before cutting it into strips and allowing it to equilibrate for several hours.

Finally, the blank data show that the carbon dioxide cleaning reduced the background of the target analytes in the paper, but that, for the PCBs, these levels are still unacceptably high (Table 2).

Table 2. Background levels of target analytes in the paper blanks analyzed in the second validation effort.

ANALYTE	MEDIAN	AVERAGE	SD	RSD	% OF BLANKS PREDICTED TO BE ABOVE THE LOWER CALIBRATION LIMIT
	pg/µL	pg/µL	pg/µL	%	
γ-HCH (Lindane)	0.002310	0.001958	0.000794	41%	0%
PCB-101 22'455'-PeCB	0.134	0.143	0.047	33%	100%
PCB-105 233'44'-PeCB	0.068	0.073	0.024	33%	99%
PCB-138 22'344'5'-HxCB	0.156	0.162	0.062	39%	99%
PBB-153 22'44'55'-HxBB	0.000	0.000	0.000	NA	0%

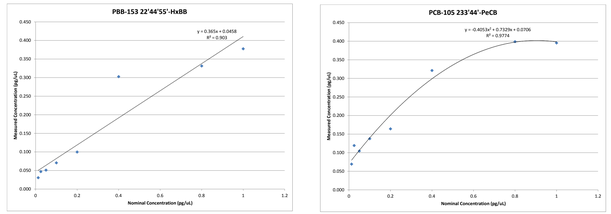

Figure 2. Curves showing measured concentration versus nominal concentration. For PCBs, the response is non-linear, while it is linear for PBB-153. Both curves, however, show less than the nominal concentration being measured.

CONCLUSIONS

While the technique shows promise, analysis of dried blood spots for POPs has some significant hurdles to overcome. In particular, the failure to recover all of the target analyte spiked into the blood and the PCB background in the paper represent the biggest obstacles to successful implementation of the technique.

We are continuing to explore the unexpectedly low measured concentrations. These results suggest that integration of the labeled standard spike with a dried blood spot represents a special analytical challenge that will require additional effort to overcome. Possible avenues to resolution of this problem include digestion with carboxypeptidase or some means of disaggregating protein clusters formed during the drying of the blood.

Removal of background PCBs may require successive cleaning steps, as no single step appears to have removed all of the PCBs present. Even the solvent blank generated in the laboratory showed some PCB background. Elimination of this background might require a number of procedural changes. Fortunately, the samples did not show a need for cleanup, which can often introduce more PCBs to the process.

Sonication may not be an adequate extraction procedure and Soxhlet extraction of a similar technique may prove more efficacious in recovering target analytes and reducing background.

In spite of these various drawbacks, the detection limits necessary to analyze POPs in dried blood spots can be achieved together with a linear response and quantitation. The instrumental technique necessary, then, can be developed and validated. The hurdles that remain are preparative in nature. Now that they are better understood, they, too, may be overcome.

REFERENCES

1. Lu D, Wang D, Ip HSS, Barley F, Ramage R, She J; (2012) *Journal of Chromatography* B 891-892: 36-43

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