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NOVEL SOLID-PHASE EXTRACTION TECHNIQUES FOR BIOLOGICAL AND ENVIRONMENTAL ANALYSIS USING ISOTOPE DILUTION MASS SPECTROMETRY

A Dissertation

Submitted to Bayer School of Natural and Environmental Science

Duquesne University

In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

By

Andrew J. Boggess

May 2015

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NOVEL SOLID-PHASE EXTRACTION TECHNIQUES FOR BIOLOGICAL AND ENVIRONMENTAL ANALYSIS USING ISOTOPE DILUTION MASS SPECTROMETRY

By

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ABSTRACT

NOVEL SOLID-PHASE EXTRACTION TECHNIQUES FOR BIOLOGICAL AND ENVIRONMENTAL ANALYSIS USING ISOTOPE DILUTION MASS SPECTROMETRY

By

Andrew J. Boggess

May 2015

Dissertation supervised by Professor H.M. Skip Kingston

Awareness and study of the ways in which the environment can interact with the personal genetics and epigenetics of an individual has grown substantially in recent years, resulting in the field of Exposomics. In an era of increasingly personalized medicine, novel techniques are necessary to ensure the accurate and sensitivity measurement of clinically and environmentally relevant molecules in biological and environmental samples. Addressing existing shortcomings cited in literature, methods were developed and optimized for the extraction, separation, mass analysis, and quantification of a suite of environmental organic pollutants in both biological and environmental samples, with the primary objective of improving accuracy, increasing sensitivity, and reducing sample and reagent consumption. The secondary objective of this research was the production of validated methods capable of inter-laboratory method transfer with minimal training required in the receiving laboratory. Two novel methods have been developed, optimized, validated, and applied to collaborative environmental research. These novel methods

represented a demonstrative improvement upon existing methods in both analytical quality and capability for inter-laboratory method transfer. Both developed methods were utilized in two collaborative clinical research studies investigating the impact of environmentally-sources agents on children diagnosed with autism spectrum disorders (ASD). The high-quality data obtained in these studies yielded results that may have provided valuable insight into the development and maintenance of autism spectrum disorders. These novel methods allowed for the discovery of a first-of-its-kind variable in the children with ASD, compared with controls. This variable was statistically predictive for the probability of an individual being diagnosed with the most behaviorally severe autism disorder, with a statistically significant overall model fit. This novel analytical method was then expanded in breadth through application to industrial and municipal wastewater to aid in updating EPA Method 625 for wastewater analysis. Applying this novel method to wastewater produced data of higher analytical quality, in both accuracy and precision, compared with all other collaborative laboratories. The methods developed in this work for the quantification of organic molecules implicated in environmental human health in biological and environmental samples have significantly improved analytically upon existing methods and have yielded clinically relevant findings in collaborative clinical research studies.

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Chapter 1: Introduction

1.1 Exposome and Exposomic Research

Emergent data has implicated environmental exposure, combined with genetics, as a potential causative factor in the development of numerous disease states.[1-5] The U.S. Centers for Disease Control and Prevention and the U.S. National Institute for Occupational Safety and Health have endorsed the term "exposomics" to foster an increased understanding of how environmental exposure can interact with personal genetics, physiology, and epigenetics to impact overall health.[6] A link has been suggested between serum concentration of certain organic pollutants and etiology of specific disease-states such as autism spectrum disorders, heart disease, diabetes, obesity, and lupus, among many others.[1,3,7-11] Taken collectively, these carbon-based toxins are known as persistent organic pollutants (POPs) and exhibit common characteristics such as semi-volatility, environmental persistence, low water solubility and inherent toxicity.[12]

In 2001, 178 countries and the European Union signed the Stockholm Convention on POPs, which restricted or eliminated the production of certain chlorinated pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and chlorinated benzene compounds.[13, 14] The World Health Organization has developed guidelines for quantifying POPs in biological fluids to assess exposure.[15] Increasing interest has been placed in the development of methods to quantify POPs in serum and other biological and environmental matrices for the purposes of improving environmental human health and disease diagnosis and prevention.[16-18] Understanding and defining the potential link between exposure to environmental contaminants, such as POPs, and the individualized health of human beings is a primary goal of exposomic research.[19-21]

One of the most studied disease-states in exposomic research is autism spectrum disorders (ASD).[22, 23] ASD are characterized behaviorally by impaired social communication and interaction, and repetitive behaviors. [24] Recent studies have suggested the incidence of ASD is increasing, [25, 26] but scholarly opinion remains divided on the mechanism governing the increase. [27] While studies have estimated that at least half of the causation of ASD is attributable to genetic factors, [28] it has been suggested that increased risk of ASD-diagnosis may be associated with increased exposure to environmental triggers. [29, 30] These studies have correlated maternal exposure to environmental air pollutants and proximity to coal-fired power plants, pesticide-rich agricultural fields, known toxic-chemical sites, and traffic-related air pollution with increased rates of ASD. [31-36] Noting that environmental exposures typically occur in combination, not isolation, researchers have recommended that greater focus be placed on examining the combinative physiological effects of organic pollutants. [37]

Figure 1.1 demonstrates the complex relationship that environmental pollutants have with the systems of the human body.[38-41] One of the objectives of exposomics is to bridge the many disparate fields that separate the study of the numerous bodily systems to gain a more detailed view of the interactions between the human body and its environment.

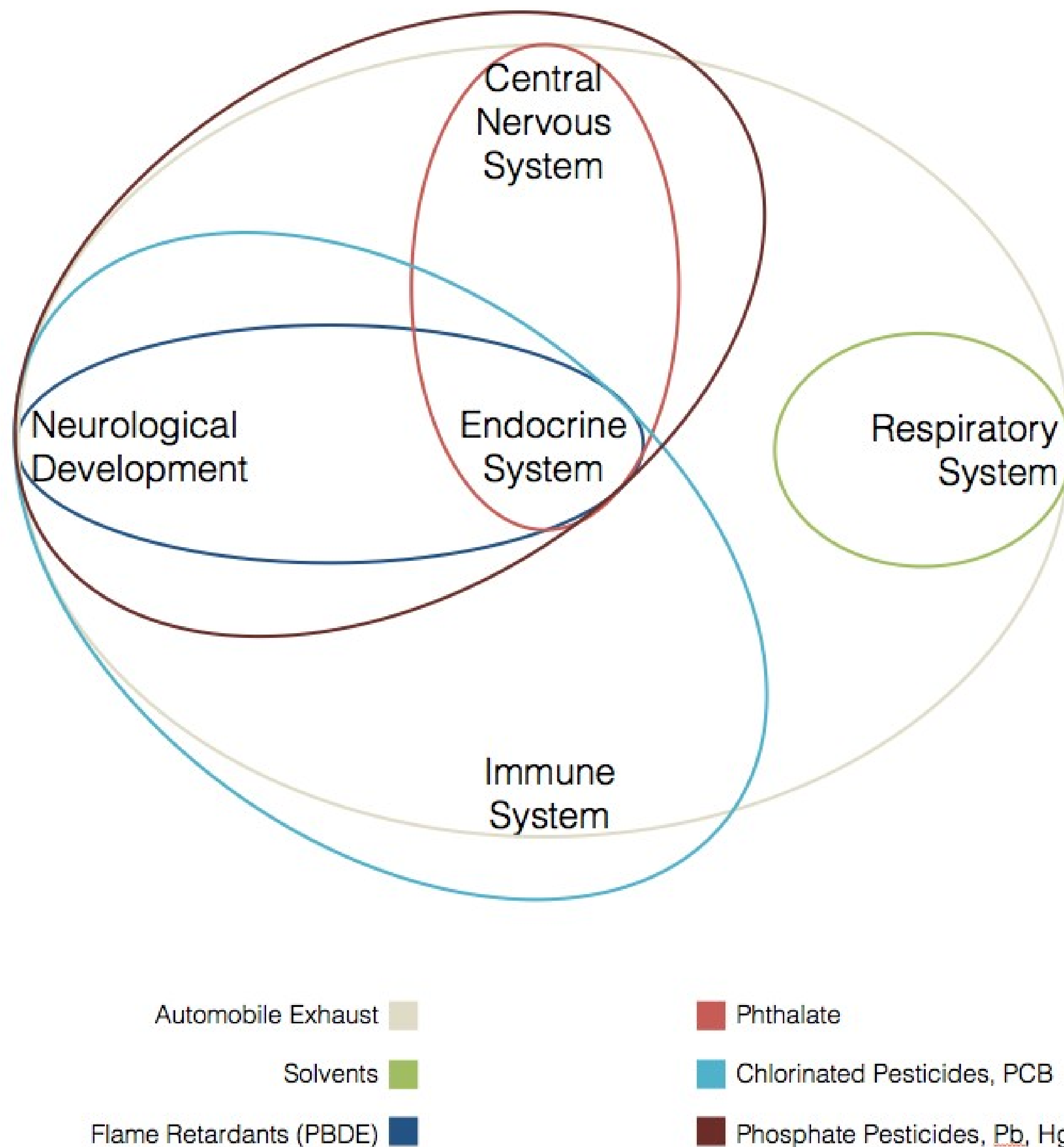

Figure 1.1:[38-41] Demonstration of the interconnectivity of bodily systems and the broad effects of common environmental pollutants

Beyond simply studying the effects of environmental pollutants, exposomic researchers have focused on sourcing exposures, which has lead to the inter-field collaboration of many differing research interests. In addition to biological fluids, researchers have studied such disparate exposure routes as drinking water, ground water, air, soil, food, and household environments. The isolation of an individual from his or her environment also has been used to

gauge the environmental effects on particular disease-state populations. Waters, particularly drinking and wastewaters, are of interest to exposomic researchers, as these aqueous solutions possess many similar chemo-physical characteristics as biological fluids.

Methods have been developed, and continue to be developed, to extract and quantify decreasing concentrations of harmful environmental pollutants in biological fluids. In an era of increasingly personalized medicine, it is reasonable to suggest that the prevalence of clinically standard analytical tests for environmental pollutants will only increase as researchers continue to learn more about the impact on human health caused by exposure to environmental pollutants.

1.2 Inter-Laboratory Analytical Method Transfer

It may not be feasible to expect all commercial, government, clinical, or academic laboratories to reproduce validated, peer-reviewed methods to the same degree of high analytical quality as the developing laboratory. To ensure that quality control parameters are met by the method-receiving laboratories, regulatory bodies have instituted quality assurance / quality control guidelines for inter-laboratory method transfer. The U.S. Pharmacopeia describes the most common type of analytical method transfer: transfer of a validated method from a developing laboratory (transferring laboratory) to an outside laboratory (receiving laboratory).[42] The most common method for validating the analytical quality of the receiving laboratory is by comparative testing. Comparative testing is performed by the receiving laboratory by the analysis of a sample intentionally prepared at a known concentration of select analyte(s) by the transferring laboratory. Quantification of the prepared sample by the receiving laboratory to pre-determined analytical quality (often measured by accuracy, precision, sensitivity, and selectivity) is required to validate the QA/QC of the receiving laboratory. Further, the U.S. Food and Drug

Administration stipulates that the transfer validation must by verified under actual laboratory conditions, including the sample matrix, instrumental parameters, and analysts that be present when the actual samples are analyzed.[43]

To ensure the adoption of developed high-quality analytical methods by outside laboratories, it is important to limit the number of highly complex sample preparation steps and to reduce the influence of analyst skill on final quantitative quality. Much of the research described herein focused on producing methods capable of being replicated by outside laboratories with only minimal additional training. This objective was accomplished using extraction technologies and quantitative procedures that improved sensitivity, selectivity, and reproducibility to correct for many common sources of analytical error.

1.3 Isotope Dilution Mass Spectrometry

Quantification by isotope dilution mass spectrometry (IDMS) is based on spiking of known amounts of enriched isotopic analogs of each compound into a sample. Prior to extraction, equilibrium must be obtained between the endogenous and labeled isotopic compounds. With the known isotopic abundance of both endogenous and isotopically labeled analytes, the amount of spike added to the known amount of sample, concentration of the spike added, and altered isotopic ratio, the concentration of the endogenous molecule in the sample can be directly calculated.[44] The method of IDMS eliminates calibration curves and correction factors by avoiding internal standards that are not chemically identical to an analyte of interest. In this way, IDMS quantification is based on a direct mathematical comparison, not external calibration. Equation 1 is an example of expressing the concentration that is derived from the known data and the ratio measurement from the mass spectrometer.[44]

$$\text{Concentration, µmole/g} = [(C_sW_s/W_x) \times {}^iP_s-(R_{i/n} \times {}^nP_s)] / [(R_{i/n} \times {}^nP_x) - {}^iP_x] \quad \textit{eq. 1}$$

$R_{i/n}$ = Peak area of isotopically enriched molecule / peak area natural molecule

Where, Cs is the concentration of the isotopically labeled analog of the target anlayte, Ws and Wx are the masses of the isotopic analog spiked into a sample and the natural sample (respectively), iPs and iPx represent the fraction of the target analyte in the isotopically enriched form in the isotopic spike (e.g. 0.99) and the natural sample (e.g. 0.01, respectively), nPs and nPx represent the fraction of the target analyte present in the naturally occurring form in the isotopic spike (e.g. 0.01) and natural sample (0.99, respectively).

Being chemically identical and in equilibrium in solution, the endogenous and spiked isotopes are extracted with equivalent efficiency and recovery. These chemical indistinguishable isotopically distinct analytes create an advantage of IDMS compared with calibration curve, internal standards, and response factor quantifications. Once equilibration is achieved between endogenous and isotopically labeled compounds, IDMS can mathematically correct for many of the sources of error and variance associated with extraction, mass spectrometry, and quantification. These sources of error include, among others, imprecise sample preparation, poor extraction reproducibility, low analyte recovery, instrumental drift, sample loss, and physical or chemical interferences. Thus, IDMS reduces the contributions of random and analyst errors to overall quantitative quality, resulting in greater reliability and uniformity of accuracy and precision. The IDMS quantitative method is described in a standard government method, U.S. EPA Method 6800, Revision V.[44]

1.3.1 Mass Bias Factor

A mass bias factor must be determined experimentally to mathematically correct for the differences in ionization between the natural and isotopic forms of an analyte in samples at identical concentrations. A ratio is computed representing the signal intensity of a natural compound as a function of the signal intensity of the isotopic analog at identical concentrations.[45] This mass bias factor, M.B., is applied as a correction factor to the isotope signals in the traditional IDMS equation, as given by the following equation:

Concentration, μmole/g = $M.B. \times [(C_s W_s/W_x) \times {}^{i}P_s - (R_{i/n} \times {}^{n}P_s)] / [(R_{i/n} \times {}^{n}P_x) - {}^{i}P_x]$ *eq. 2*

$M.B. = A_N C_i / A_i C_N$

Where A_N and A_i are the signal obtained from the TOF-MS for the natural analyte and the isotopic analog, respectively. C_N and C_i are the concentrations of the natural analyte and isotopic analog, respectively. Mass bias factors were computed for each compound and used as an internal correction factor in the quantitative method.

1.4 Solid-Phase Extraction

Solid-phase extraction (SPE) is a sample cleanup and pre-concentration procedure to isolate analytes of interest from potentially interfering or harmful biological compounds using primarily hydrophobic interactions and cationic or anionic exchange.[46] SPE extracts compounds from a mobile phase by taking advantage of the selective affinity. Reverse-phase SPE utilizes a stationary phase (typically in either a column or cartridge form) of linked hydrocarbon packing to selectively extract hydrophobic compounds from aqueous solutions. Normal-phase SPE uses a

polar stationary phase to isolate polar compounds from hydrophobic contaminants in a stationary phase. SPE is often used on aqueous and biological samples to selectively extract and pre-concentrate analytes prior to analysis by LC/MS or electrospray ionization (ESI) TOF-MS. Figure 1.2 demonstrates the procedure for isolating compounds of interest from solution and contaminating agents in column pass-through scenario.

When applicable, SPE is often used in place of traditional liquid-liquid extraction due to the increased selectivity afforded by SPE and the reduction in solvent consumption and waste generation. SPE has been in use since the 1960s and has given rise to newer stationary phases and extraction procedures.[47] Two of the most commonly used SPE technique, in addition to traditional column and cartridge, are solid-phase microextraction and stir-bar sorptive extraction.

1.4.1 Solid-phase Microextraction

Where column and cartridge SPE extractions rely on so-called "pass-through" extractions in which a mobile phase is passed through a stationary phase, solid-phase microextraction (SPME) was one of the first extraction techniques to rely on passive interaction with a stationary phase.[48] This passive interaction could occur via immersion into a solution or suspension into the headspace of a sample. Traditional SPME uses a flexible metallic fiber coated in a hydrophobic stationary phase, typically made of polydimethylsiloxane (PDMS). Where SPE reduced solvent consumption by eliminating liquid-liquid extraction, SPME further reduces solvent consumption by utilizing a completely thermal desorption procedure. After exposure of a PDMS-coated fiber to a sample, the adsorbed analytes are then desorbed directly into the carrier gas flow in a heated inlet of a gas chromatograph (GC). In this way, SPME can eliminate solvent consumption associated with sample extraction.

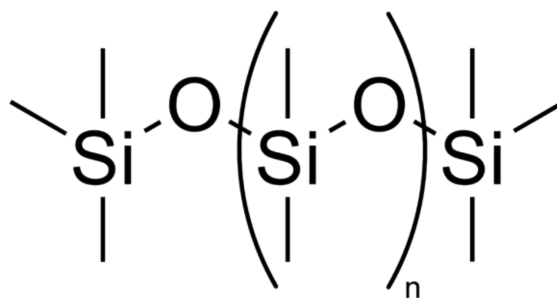

Figure 1.2: Chemical structure of PDMS

The mechanism of SPME extraction is significantly more complex than of traditional SPE column or cartridge extraction. The following equation represents the amount of an analyte (n_f) present in the stationary phase following SPME extraction:[49]

$$n_f = c^o{}_s V_f V_s K_{fs} K_1 / (K_{fs} K_1 V_f + K_1 V_g + V_s) \qquad \textit{eq. 3}$$

Where, C_s is the concentration of the analyte in solution, V_f is the volume of extraction phase PDMS, V_s is the volume of solution, K_{fs} is the analyte-specific partition coefficient between PDMS and aqueous phase (typically approximated by the more-standard K_{ow}), and K_1 is the analyte-specific partition coefficient between gas phase and solution phase. The extraction mechanism is demonstrated in figure 1.3, showing the passive nature of SPME extraction. Due to the passive nature, many analytes require up to 24 hours of exposure to a sample to obtain quantitative extraction.

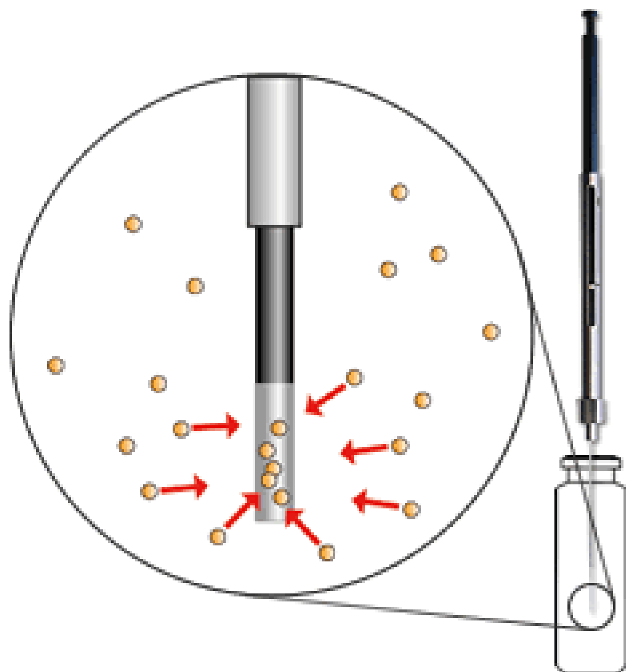

Figure 1.3: Passive extraction of an analyte from an aqueous solution using SPME

Due to mechanism limitations of attaching the PDMS stationary phase to a flexible metallic fiber, the PDMS coating must be attached by epoxy. This means that excessive exposure of the SPME fiber to an organic solvent will dissolve the epoxy and cause mechanical failure of the SPME fiber. Because of this, nonvolatile compounds (which cannot be removed thermally) may irreversibly bind to the SPME fiber and degrade the analytical quality of a fiber. There have been methods proposed in literature to permit immersive analysis in samples containing nonvolatile compounds, such as whole blood and blood serum.

1.4.2 Stir-bar Sorptive Extraction

Stir-bar sorptive extraction (SBSE) is a solventless-desorption alternative to traditional solid phase extraction (SPE)[50] that is rapidly gaining application in analytical laboratories. SBSE utilizes a glass-coated iron core wrapped in a polymeric extraction phase and functions by the same extraction mechanism as the industry-accepted SPME.[48] In addition to a 50 – 100 fold

increase in extraction phase volume compared with typical SPME,[51] SBSE also changes the extraction mechanism from a passive diffusion-based adsorption to active sampling by rapidly stirring an analytical sample.[52] Most SPE tools (e.g. cartridges, disks, filters, fibers) are incompatible with the analysis of biological fluids without significant sample preparation and cleanup. In contrast with SPME fibers, SBSE bars are not produced with epoxy; rather the PDMS stationary phase is attached mechanically. This means that SBSE may be conducted in complex solutions containing nonvolatile compounds that would otherwise cause irreversible damage to SPME fibers. SBSE bars can be rinsed with small volumes of methanol to remove any fouling agents to avoid stationary phase damage. Figure 1.4 shows a simple schematic of the most common type of stir bar used in SBSE.

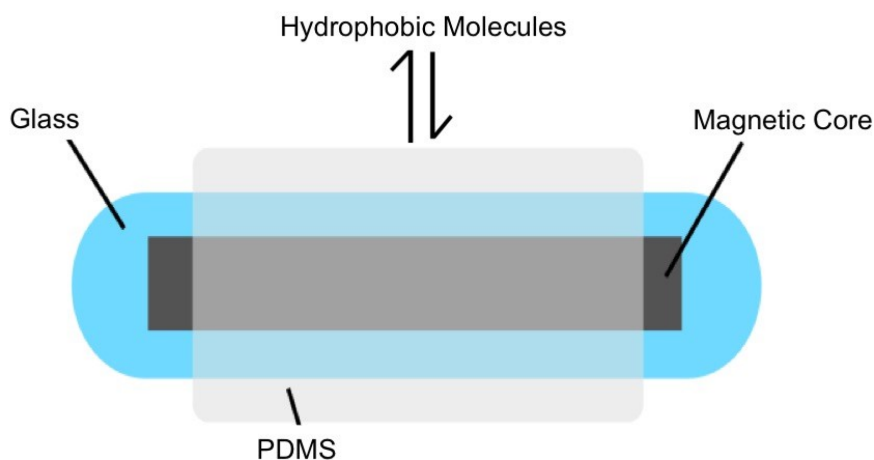

Figure 1.4: Schematic diagram of a typical stir bar used in SBSE

For the extractions of analytes of mixed-characteristics, dual SBSE has typically been used as a tandem-in-time extraction.[53] For mixed volatility analytes, dual SBSE is conducted by immersive and headspace analysis simultaneously.[54] Following stir-bar extraction, analytes are thermally desorbed from the bar(s) in the inlet of a gas chromatograph (GC). The relative cost difference between sorbent stir-bars and other SPE tools means that for SBSE, more than other

SPE methods, the efficient use of a limited number of stir-bars is often the limiting factor in overall processing time.

According to the research performed by Baltussen et al., SBSE can theoretically recovery a quantitative amount of a wider range of analytes, compared with SPME.[55] The larger volume of PDBS present in the SBSE bars should allow for the extraction of greater amounts of typically poorly extracting compounds (i.e. compounds with a log $K_{o/w}$ of 4 and lower).

The most common technique for sample analysis using SBSE is thermal desorption into a gas chromatography / mass spectrometer (GC/MS). Volatilizing the adsorbed analytes from the stir-bar into the carrier gas stream of the GC/MS eliminates solvent from the desorption process. As not all compounds will volatilize instantly, a liquid-nitrogen cryo-trap is typically installed beneath the thermal desorption device.

1.5 Relevance to Research

Technologies have been developed to sensitively extract and accurately quantify trace-levels of environmental pollutants in biological and other aqueous solutions. It was the objective of this research to combine the relatively simplistic approach of SPE, SPME, and SBSE, with the high accuracy and precision afforded by IDMS to produce analytical methods that significantly decreased solvent consumption, provided accurate and precise quantification, and enabled high-quality inter-laboratory method transfer for the analysis of biological and environmental samples.

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Chapter 2: Solid-phase microextraction for the analysis of volatile and semi-volatile compounds in serum

2.1 Introduction

Numerous methods exist to extract and quantify organic pollutants in biological fluids. A standard, accepted method for the extraction of organic, hydrophobic analytes from aqueous solutions is by SPME.[1, 2] Government standardized methods and regulations have specified the use of SPME analysis since the 1990s.[3, 4] Because of its wide acceptance and broad research capacity, SPME was chosen as the extraction technique for the development of a method for analysis of a range of organic pollutants in blood serum.

In clinical analysis, large volumes of sample are often required to attain the analytical sensitivity to produce actionable quantitative results. Due to the low environmental concentrations of many organic pollutants, researchers typically quantify these compounds in milliliter volumes of whole blood or blood serum,[5, 6] with 0.5 mL considered an exceptionally low volume.[7] However, for some patients (particularly children and immune compromised) large volumes of blood are difficult to acquire, especially if taken in conjunction with additional blood tests. While it is not commonplace for medical practitioners to recommend organic pollutants in typical blood tests, the general trend toward personalized medicine may one day make this type of analysis routine.[8]

Many organic pollutants can be classified as persistent organic pollutants (POPs) based on characteristics such as low volatility, environmental persistence, and inherent toxicity.[9] Many studies have been conducted to investigate both the acute and chronic health effects of exposure to many of these POPs.[10, 11] These studies were collected and used to develop a list of the most

commonly researched POPs and their peer-reviewed potential for human health effects. Figure 2.1 demonstrates the structural similarities between these chosen analytes.

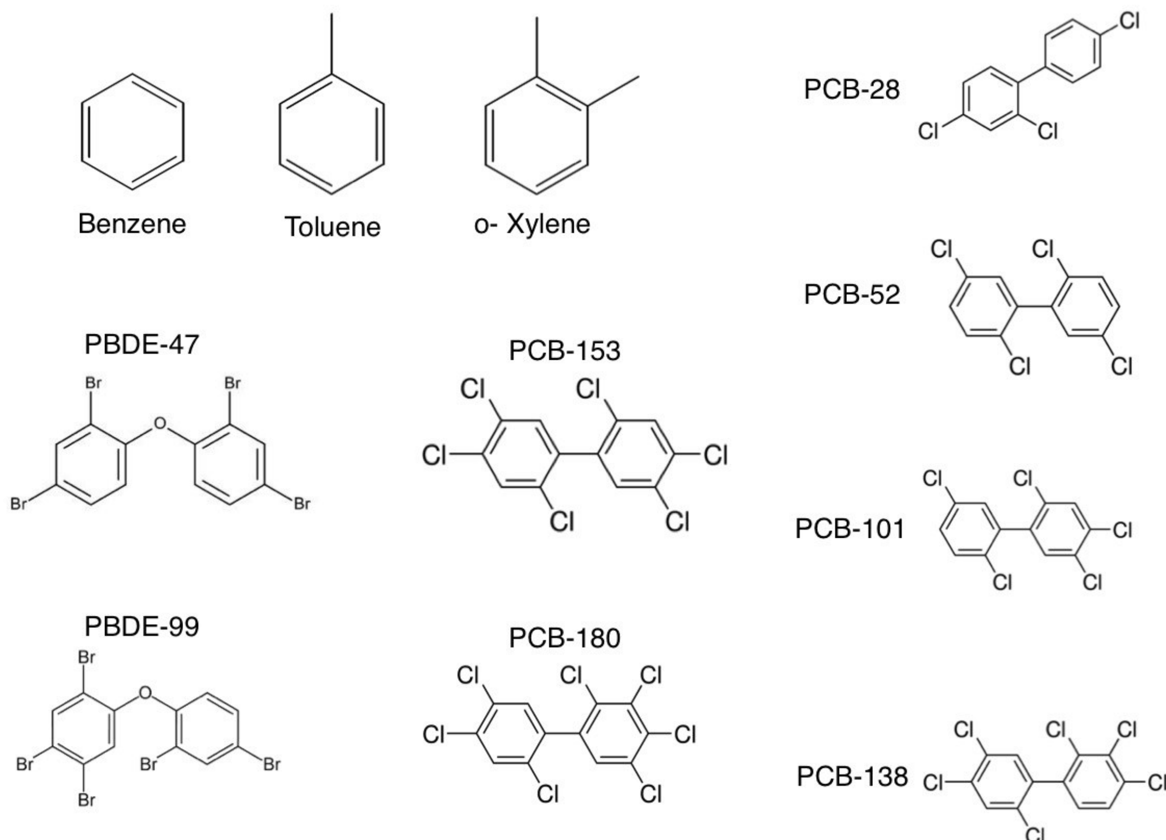


Figure 2.1: Structural similarities between selected POPs implicated in human disease states and etiology

The purpose of this study was to develop an analytical method capable of matching and exceeding the analytical quality and sensitivity of existing methods for the extraction and quantification of semi-volatile and volatile POPs in blood serum, while reducing the sample volume to 200 µL. It was hypothesized that the imprecision and irreproducibility of SPME as the limit of quantification (LOQ) is approached could be reduced by the addition of IDMS quantification in place of calibration curves. This developed method would then be further optimized for immersive analysis of nonvolatile analytes. The developed and optimized method

would be validated against certified concentrations of the selected POPs in blank-subtract blood serum. Once validated, this method would be applied to a collaborative health study investigating the exposure of ten children with clinician-diagnosed autism to environmental POPs. Further, this validated method would be used to assess the effectiveness of a sleeping cleanroom on the ability of these children to detoxify the selected POPs.

2.2 Materials and Methods

2.2.1 Reagents

Benzene, toluene, and o-xylene standards (99.9% pure) were purchased from Sigma-Aldrich (Saint Louis, MO). Benzene-d6 (99.6% label purity), toluene-d8 (99.6% label purity), and o-xylene-d10 (99.6% label purity) were purchased from C/D/N Laboratories (Pointe-Claire, Quebec, Canada). A standard mixture of six unlabeled PCB compounds, IUPAC congeners 28, 52, 101, 138, 153, and 180 (99.8% pure), in isooctane were purchased from Cambridge Isotope Laboratories (Tewksbury, MA) certified in a NIST-validated laboratory. Solutions of $^{13}C_{12}$ -labeled analogs (99% label purity, 99.8% chemical purity) for each PCB compound in nonane were purchased from Cambridge Isotope Laboratories.

2.2.2 Instrumental Methods

An automated GERSTEL MultiPurpose Sampler II with thermally controlled agitator and automated SPME holder, Agilent 7890A Gas Chromatography, and Agilent 5975c single quadrupole mass spectrometer were used for sample analysis. The SPME fiber used contained a mixture of PDMS and divinylbenzene (DVB) polymers. A 5%-phenyl polydimethylsiloxane (PDMS) GC column was used (30m x 0.25mm x 0.25mm) for chromatographic separation

(Agilent DB-5ms). The quadrupole mass spectrometer was operated in select ion mode, set to the determined quantitative and confirmatory ions for the VOC and PCB compounds, both isotopically labeled and unlabeled. Agilent Chemstation and Masshunter were used for data analysis.

For development, validation, and application, isotopic analogs spikes were added by mass to all sample replicates and sample vials were vortexed for 5 minutes to ensure equilibration and homogenation among natural and isotopic molecules. .Samples containing 200 μL of blank-subtracted blood serum and isotopic analog spikes of all analytes of interest were extracted using SPME for 15 minutes with a a sample agitation of 700 rpm. The automated sampling system transferred the SPME fiber to the GC inlet to undergo thermal desorption at 300 °C into the carrier gas stream. Following chromatographic separation, analytes underwent electron ionization in the mass spectrometry ion source and mass analysis by single quadrupole spectrometry. For peak identification and integration, the Agilent Agile integration algorithm was used, with a minimum peak height of 1000 mass spec counts. To assure reproducible integration results between replicates, the automated Agile integrator was used for all peak area calculations. Demonstration of peak area automated calculation can be found in figure 2.2. The Agile integration algorithm recognizes a peak as any point at which the chromatogram baseline exceeds 1000 units of peak height. The Agile integration algorithm sets the left and right retention-time bounds as the points at which the peak height falls below 1% of the total peak height. Then an integration is performed and peak area, in arbitrary units of mass spec counts, is calculated automatically.

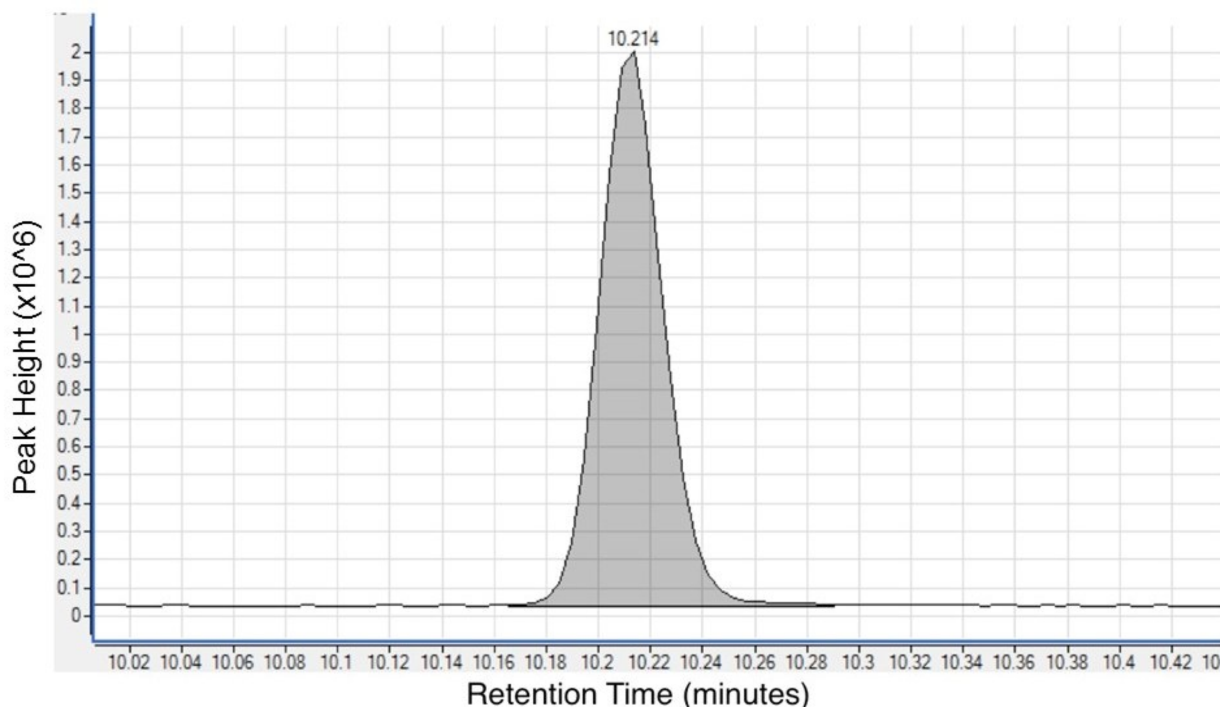


Figure 2.2: Demonstration of automated peak area calculation by the Agile integrator, bounding the starting and ending retention times based upon defined limits of peak height

An air-filtration system was installed following method development procedures. The filtration system consisted of a Plexiglas enclosure surrounding the GC/MS, sample storage racks, and automated sample-handling robot. The enclosure was sealed at the edges using non-VOC sealant and the exposed aluminum surfaces of the solid frame were sealed using non-gassing, non-VOC, and phthalate-free cleanroom paint. Sealed to the top of the enclosure was an air pump that forced laboratory air through an activated carbon sheet and a high-efficiency particulate air (HEPA) filter before blowing over the instrumental system. The filter enclosure system was not sealed at the bottom. In this way, the enclosure was designed to maintain positive air-pressure and prevent laboratory air from flowing back into the enclosure.

2.2.3 Analyte Loss Via Septa Puncture

A sealed, spiked serum sample was placed inside of a sealed metal canister with a strip of activated carbon suspected in the headspace of the metal container. The canister was placed in an oven at 70 °C for three hours. A second sealed, spiked serum sample was placed into a sealed metal canister with a strip of activated carbon suspended in the headspace and a SPME fiber was placed through the septum of the seal vial and remained in the septum throughout the experiment. This canister was placed in an oven at 70 °C for three hours. Replicates (n=3) were performed for the sealed sample and the SPME-pierce sample. Analysis was conducted by placing the activated carbon strip into a vial with 5 mL of hexane and allowed to equilibrate for 1 hour. A 50 µL sample of this extraction phase was directly injected into the GC inlet and a comparison was generated between the sealed sample vials and the SPME-pierced sample vials.

2.2.4 Method Development and Optimization

For all method development, optimization, and validation experiments, samples containing 200 µL of blank-subtracted serum were spiked with certified concentrations of an analyte of interest. Blood serum was brought to room temperature from a -80 °C freezer and added by mass to a 2 mL GC vial. A stock solution of an analyte of interest at a certified concentration in a water-miscible solvent was then added to the vial by mass, to achieve a designated final concentration for the analyte of interest.

Extraction time, extraction temperature, desorption time, desorption temperature, and cryotrap temperature were optimized experimentally for every analyte, in that order. These parameters were optimized using solutions prepared using samples containing all analytes of interest simultaneously. For extraction time optimization, a solution containing 10 µg/g of VOC

and 10 ng/g of PCB compounds was extracted for 5, 10, 25, and 45 minutes and analyzed using a validated literature method. The extraction time producing the highest analyte recovery (assessed as the time-point which produced the largest relative peak area, or the point at which the analyte recovery plateaued, for the largest number of analytes) was then used to assess extraction temperature using a similar procedure at temperatures of 23, 35, 50, 70, and 90 °C. The optimized extraction time and temperature was then used to assess desorption time (5, 15, 25, 45, and 60 minutes), desorption temperature (150, 200, 250, 275, and 300 °C), and cryotrap temperature (-150, -100, -50, 0, and 10 °C) in that order.

2.2.5 Method Validation

Samples were prepared by mass containing 200 µL of blank-subtracted serum in 2 mL GC vials. Certified concentrations of benzene, toluene, o-xylene, and PCBs 28, 52, 101, 138, 153, and 180 were added by mass. Similarly, benzene-d6, toluene-d8, o-xylene-d10, PCBs-13C12 28, 52, 138, 153, and 180 were added by mass to attain a final concentration of both isotopically labeled and unlabeled compounds of approximately 1450 µg/g for the VOC compounds and approximately 9.12 ng/g for PCB compounds. Samples were analyzed following the experimentally optimized method parameters found in table 2.1. Following quantification by IDMS, experimentally determined concentrations were compared at the 95% confidence level to the calculated certified concentration of the natural (unlabeled) form for each compound.

Table 2.1: Experimental optimized analytical parameters for the quantification of VOC and PCB compounds in 200 μL of serum

Parameter	Value	Parameter	Value
SPME Polymer	PDMS/DVB*	*CIS Desorption Temperature*	300 °C
Extraction Time	15 Minutes at 70 °C	*GC Column*	DB-5ms (30m x 0.25mm x 0.25mm)
SPME Desorption Time	25 minutes	*Flow Rate*	1.2 mL/min
Agitation	700 rpm	*Oven Ramp*	40 °C initial, 5 °C/min to 80 °C, 15 °C/min to 250 °C, 25 °C/min to 300 °C
Crytrap	-100 °C	*Mass Analysis Mode*	Select Ion

*A 1:1 mixture of PDMS and divinylbenzene

2.2.6 Statistics

For measurements of accuracy and precision, the percent error from calculated concentration (%error), percent relative standard deviation (%RSD), and 95% confidence intervals were used. For validation comparisons, the 95% confidence interval of the experimentally determined mean was compared with the accompanying ±5% standard deviation of the certified standard. For comparison of means, p-values were computed to compare the statistical similarity of two means with unequal variance, with $p<0.05$ indicating statistically significant difference. All analyses in blood serum were blank-subtracted using synthetic urine samples that were not spiked with either the natural or isotopically enriched analytes. Limit of quantification (LOQ) calculated as the concentration sufficient to present a chromatographic peak height equal to five-times the chromatographic baseline height.[12] Limit of detection was calculated as the analyte concentration necessary to produce a chromatographic peak height equal to three-times the chromatographic baseline height.[12] Quantification was accomplished using IDMS quantification.[13]

2.2.7 Application to Clinical Research: Autism Cleanroom Study

After obtaining approval from the Institutional Review Board (IRB) of Duquesne University (Pittsburgh, PA, USA, IRB #09-131), the cleanroom was created and the study was conducted at The Children's Institute (TCI, Pittsburgh, PA, USA). Before enrolling in the study, consent was obtained, and a pictorial document explaining the study was reviewed with each child.

2.2.7.1 Subject Enrollment and Study Design

Ten children, ages 3-12, with autistic disorder confirmed by a psychologist who is research certified to perform the Autism Diagnostic Observation Schedule (ADOS) [14] were recruited from the Neurodevelopmental Service of TCI. Eligible children were required to present low plasma zinc/serum copper ratios and abnormal T and B cell subsets. Child #2 was unable to participate in the study because of the inability to complete a blood draw. The group intentionally contained two sets of identical twins (ages 5 and 12). Children were not eligible if they had severe behavioral dysregulation, uncontrolled seizure activity, or any other severe chronic medical condition, other than autism, that required frequent management by pediatricians.

After signing the consent and being provided opportunity for child assent, each child and a parent spent two consecutive weeks for a minimum total of ten contiguous hours each night in the cleanroom between May and October 2010. During the day, the child and parent went about their normal activities and therapies. A research assistant recorded each child's sleep behaviors. The children and family's adjustment to the study was monitored by a Family Advocate

Psychologist who had no academic involvement in the study. Diet, medications, nutrients, and experiential services were held constant throughout the study.

2.2.7.2 Blood Draws and Clinical Testing

Within 24 hours before and after the two-week period of sleeping in the cleanroom, approximately 30 mL of blood was drawn at the child's home by a phlebotomist specializing in children with developmental disabilities. The blood was separated by centrifuge and aliquoted for storage at -80 °C. The Duquesne University testing included elemental analyses in serum, plasma, RBCs, and hair; total, oxidized, and reduced glutathione in RBCs; and benzene, toluene, and o-xylene along with selected polychlorinated biphenyls (PCBs) in serum. Greater detail on blood collection, processing, and storing for this work has been previously described elsewhere.[15, 16]

2.2.7.3 VOC and PCB Quantitative Methods

IDMS quantification was performed on the volatile organic compounds (VOCs) benzene, toluene, and o-xylene using methods and procedures outlined in EPA Method 1624[3] and selected PCBs (PCB-28, PCB-52, PCB-101, PCB-138, PCB-153, and PCB-180) using the modified methods and procedures outlined in EPA Method 1625 and recent peer-reviewed studies.[4, 17, 18] Separate analyses were completed for VOC and PCB quantification, each requiring 200 µL of serum spiked with ^{2}H labeled versions of each VOC or ^{13}C labeled versions of each PCB. SPME was performed on the headspace of the samples using a PDMS/DVB fiber. Replicates were performed (n=3) for each sample, both before and after the cleanroom.

2.3 Results and Discussion

Method development, optimization, and validation were performed using 200 µL of blank-subtracted serum spiked with certified standards of the analytes of interest.

2.3.1 Assessment of Analyte Loss

To obtain accuracy and maintain maximum sensitivity, volatile analyte loss over time was investigated in blank-subtracted serum spiked with 10 µg/g benzene, then frozen in a -80 °C freezer to simulate storage of blood serum samples. The change in peak area over storage-time was determined in two sets of six samples spiked: one set brought from 0 °C to 23 °C and stored until the appropriate analysis time, and one set brought from 0 °C to 2 °C and held at that temperature until the appropriate analysis time. Figure 2.3 shows the change in peak area over time of these two sets of samples. After 15 hours out of the -80 °C freezer, both sets of samples lost peak area, with the set stored at 2 °C decreasing by 25% and the set stored at 23 °C decreasing by 44%. This represents a maximum observed improvement in sample stability of 175% by storing spiked blood serum at 2 °C prior to analysis of benzene.

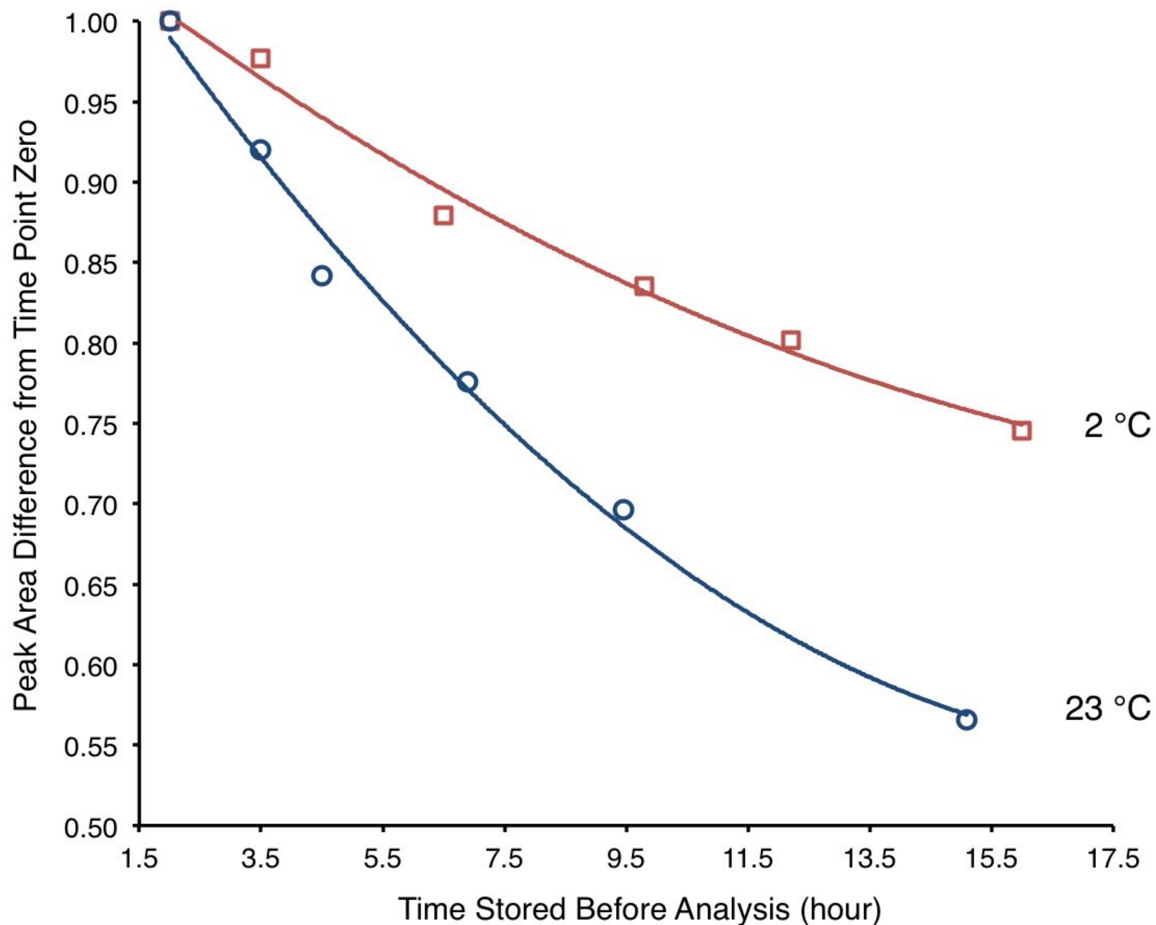


Figure 2.3: The change in peak area of two sets of serum samples spiked with 10 µg/g benzene: one stored at 23 °C and one stored at 2 °C.

Benzene was chosen as an appropriate analog, as it has the highest vapor pressure of the studied analytes. Sealed samples of spiked serum permitted loss of 10-times more benzene when heated in an oven at 70 °C with a SPME needle piercing the septum compared with sealed samples without a SPME needle in the septum. These results suggest that, while stored in the sample tray, compounds may have volatilized into the vial headspace where they leaked when the septum was pierced by the SPME needle. While IDMS quantification corrects for imprecise extraction efficiencies and sample loss, a chilled sample-tray was used to keep all serum samples at 2 °C throughout the duration of the automated sample analysis sequence to minimize VOC volatilization and subsequent analyte loss. Once spiked and equilibrated with the isotopic labeled

analogs, analyte loss affects sensitivity but does not affect accuracy, as the isotopic analog is lossed at identical ratios.

2.3.2 Assessment of Immersive SPME

A significant component to method development in this work focused on developing a process to allow immersive SPME in complex, confounding sample solutions: such as blood serum. Peer-reviewed sources provided various methods for the treatment of blood serum to permit immersive extraction without damage to either the SPME fiber or analytical instrumentation.[19] [20, 21] Following literature methods, blood serum samples were treated with a broad-spectrum serine protease, proteinase K, to digest proteins present in the serum by cleaving at hydrophobic amino acids. Literature had reported a significant increase in sample-to-sample reproducibility using this method. However, this laboratory was not able to reproduce these results. Treatment with proteinase K was found to not prevent SPME fiber fouling upon immersion into protein and lipid-containing samples.

While the original research that recommended proteinase K suggested that this treatment could prevent fouling,[19] it is likely that the instrumentation in use by Poon et al. contributed to these incorrect conclusions. The SPME fiber used by Poon et al. was not designed to retract into a metallic sheath, a very common SPME configuration today. Because of this, the primary fouling-related problem encountered by Poon et al. was a reduction in sample-to-sample reproducibility. However, modern laboratories using SPME mechanisms with retractable PDMS fibers encounter a separate problem: as proteins and lipids build up on the PDMS fiber, the irreversibly bound agents coating the fiber can “catch” on the metallic sheath and remove or strip the PDMS coating from the metallic fiber. Therefore, this laboratory was unable to reproduce or

confirm the conclusions made by Poon et al. that proteinase K can increase sample-to-sample reproducibility, these results have confirmed that this treatment does not prevent SPME fiber fouling or allow for immersive SPME analysis. This laboratory found that the SPME retractable fiber was only useful for 2 to 4 uses before physical failure destroyed the SPMS after immersion in serum.

2.3.3 Analytical Parameter Optimization

Extraction time, extraction temperature, desorption time, desorption temperature, and cryotrap temperature were optimized experimentally, in that order of priority, to achieve the greatest analyte sensitivity. Figure 2.4 demonstrates the optimization of a mixture of PCB congeners in blank-subtracted serum spiked with 10 ng/g of each PCB. Extraction efficiency plateaued for all PCB compounds after 10 minutes of extraction. The VOC analytes were optimized similarly. Experimentally optimized analytical parameters can be found in table 2.1.

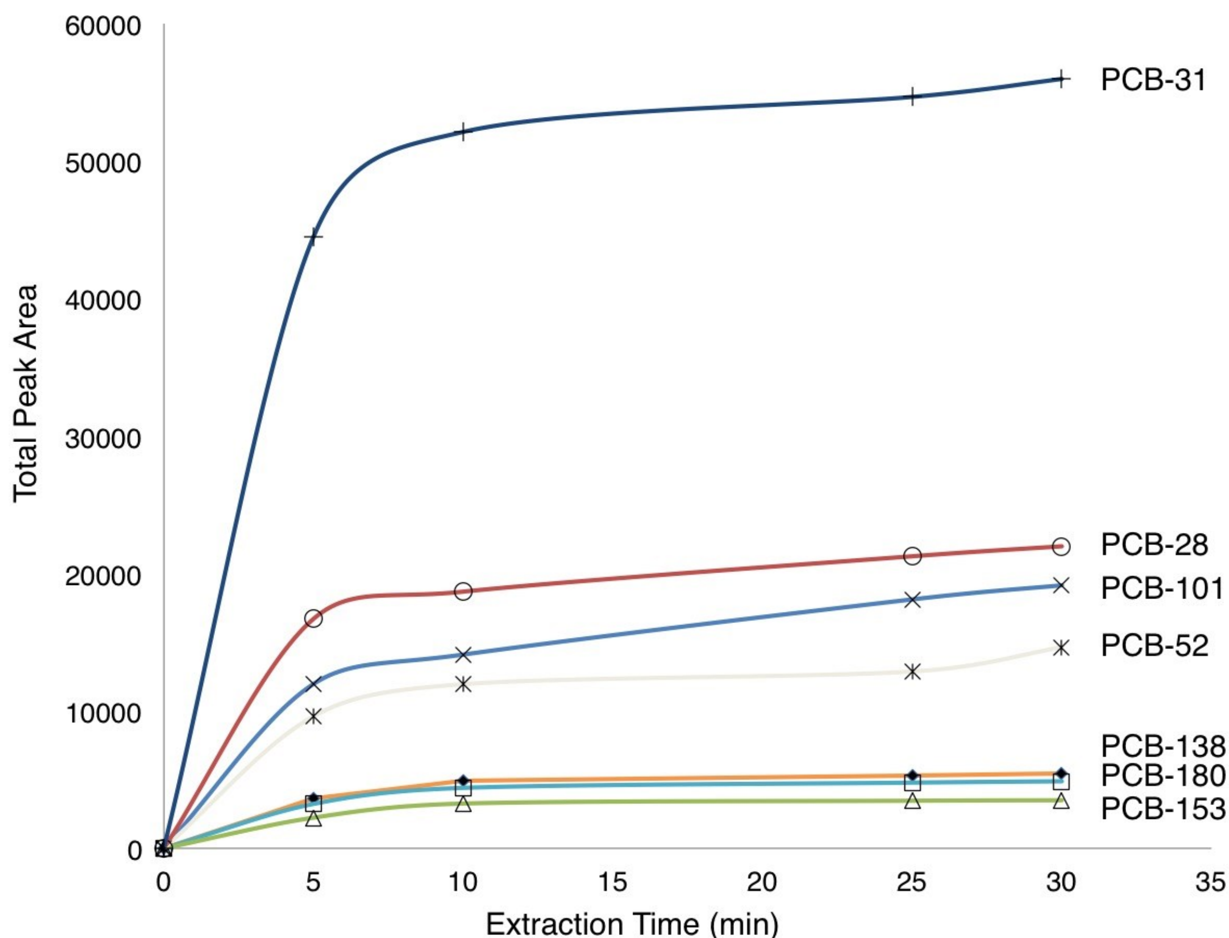


Figure 2.4: The experimental optimization of extraction time for blank-subtracted serum samples spiked with 10 ng/g of seven PCB congeners

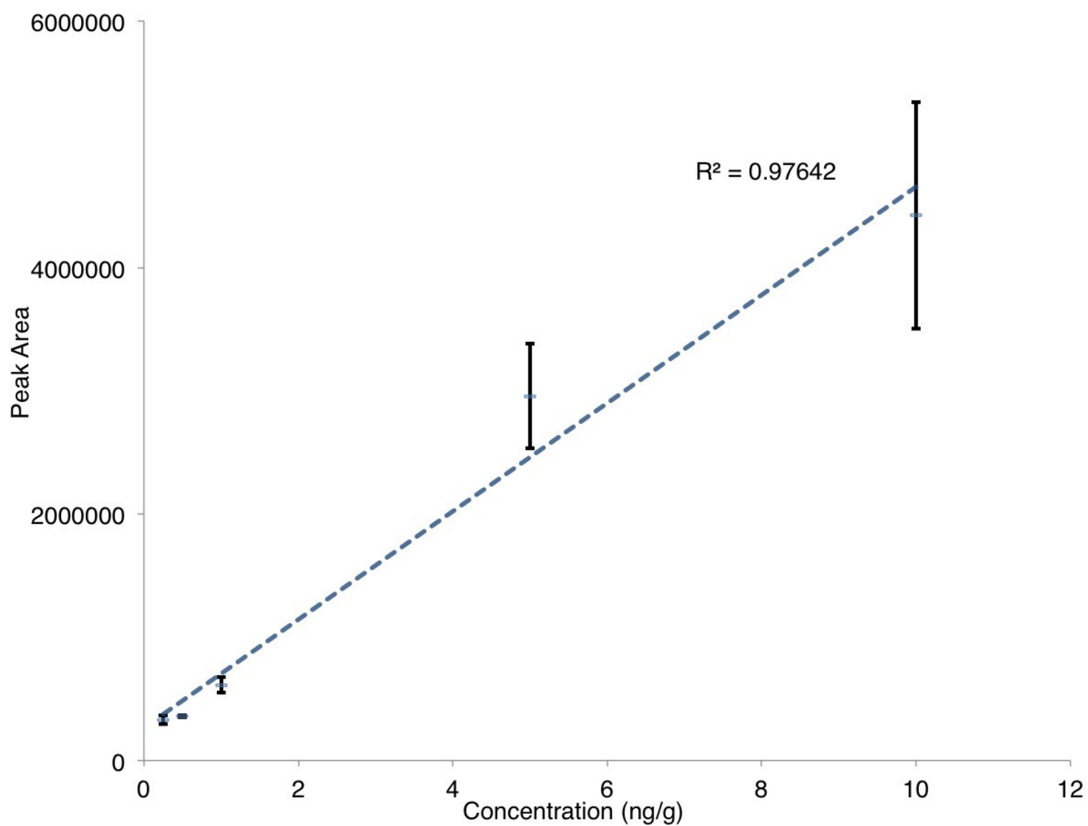
2.3.4 Method Validation

Quantification of VOC and PCB compounds was performed by IDMS, which was found to correct for much of the analytical imprecision. Analytes of interest were identified in the chromatogram using the quantitative and confirmatory peaks found in table 2.2. Isotopically labeled analytes were positively identified using the quantitative ion and retention time of the naturally occurring analog.

Table 2.2: Ions (*m/z*) for identification and quantification for the labeled and unlabeled analytes of interest

	Natural Molecule		Isotopically Labeled Molecule	
Analyte	Quantitative Ion	Confirmatory Ion	Quantitative Ion	Confirmatory Ion
Benzene	78	51	84	-
Toluene	91	65	98	-
o-Xylene	106	91	116	-
PCB-28	186	256	198	-
PCB-52	220	292	232	-
PCB-101	291	326	303	-
PCB-138	290	360	302	-
PCB-153	256	326	268	-
PCB-180	360	394	372	-

Figure 2.5 (top) shows a calibration curve generated for PCB-52 spiked into 200 µL of blank-subtracted blood serum at varying concentrations (n=3). This calibration curve yielded poor mean precision (16.2 %RSD) and linearity (R^2=0.97642). Figure 2.5 (bottom) shows the same data, with each axis adjusted to represent the ratio between naturally occurring and isotopically labeled values. With this adjustment, significant improvements were observed in both mean precision (5.12 %RSD) and linearity (R^2=0.99996).



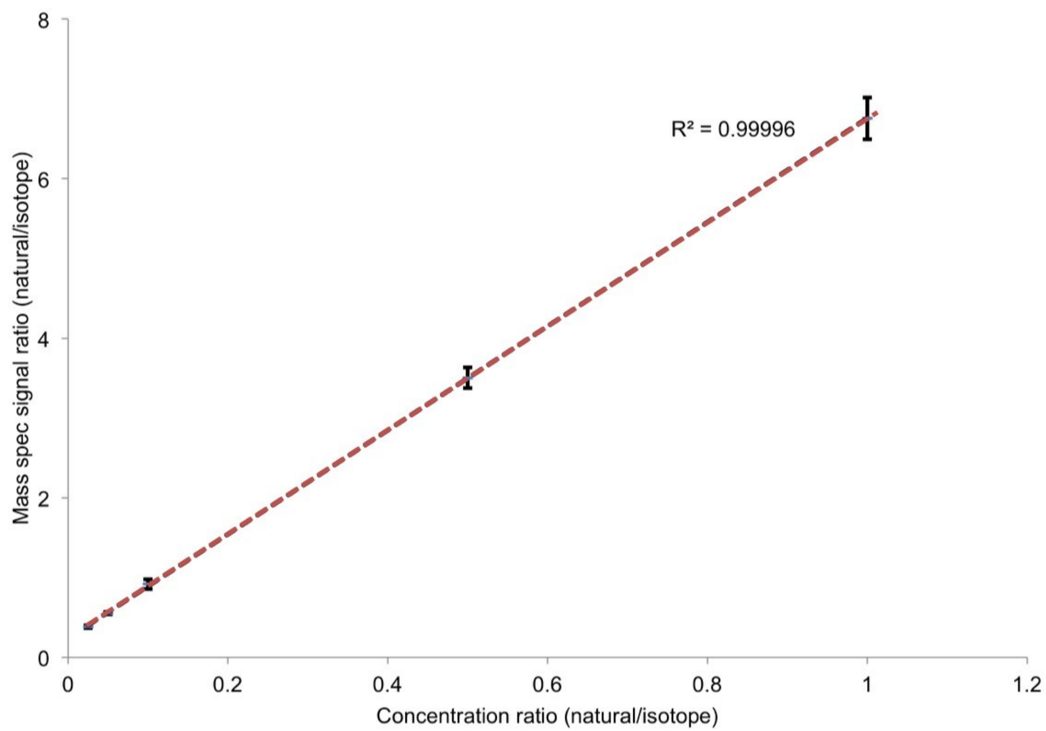
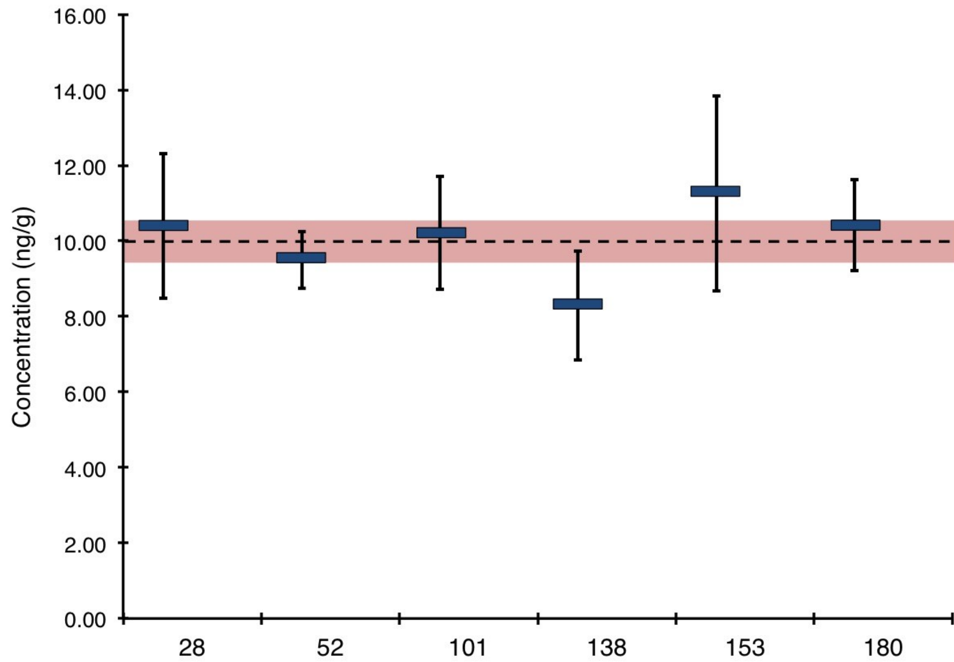


Figure 2.5: a: (top) Calibration curve generated for PCB-52 spiked into 200 μL of blank-subtracted serum at varying concentrations, and b: (bottom) the same data corrected using IDMS

Validation was performed by comparing experimentally determined concentrations with certified concentration in 200 μL of blank-subtracted serum samples using the optimized SPME-GC-IDMS method parameters. Mean quantitative accuracy exhibited 9.29% error with a mean precision of 12.1 %RSD. Limit of quantification was calculated to be 6.50 ng/g for all VOC compounds and 0.250 ng/g for all PCB compounds. The three order of magnitude difference in LOQ between the PCB and VOC compounds can be attributed, in part, to significantly higher background noise generated in the blanks at the retention times associated with benzene, toluene, and o-xylene. Validation results for each analyte of interest are summarized in table 2.3. Figures 2.6 (top) and 2.6 (bottom) demonstrate visually the overlap between the experimental and certified concentration values.

Table 2.3: Validation results for the analysis of VOCs and PCBs in 200 μL of spiked serum

	Certified Values (ng/g)		Experimental Values (ng/g)				
Analyte	Concentration	95% CI	Concentration	95% CI	|%Error|	%RSD	LOQ (ng/g)
Benzene	14.5	0.73	15.5	2.5	6.92	16	150.
Toluene	14.5	0.73	15.7	1.7	8.51	11	150.
o-Xylene	14.5	0.73	14.1	0.32	2.63	2.3	150.
PCB-28	9.12	0.46	10.4	1.9	13.9	18	0.250
PCB-52	9.12	0.46	9.49	0.49	4.06	5.2	0.250
PCB-101	9.12	0.46	10.2	1.2	11.8	12	0.250
PCB-138	9.12	0.46	8.28	1.3	9.21	16	0.250
PCB-153	9.12	0.46	10.3	3.0	12.4	20	0.250
PCB-180	9.12	0.46	10.4	1.0	14.1	9.7	0.250



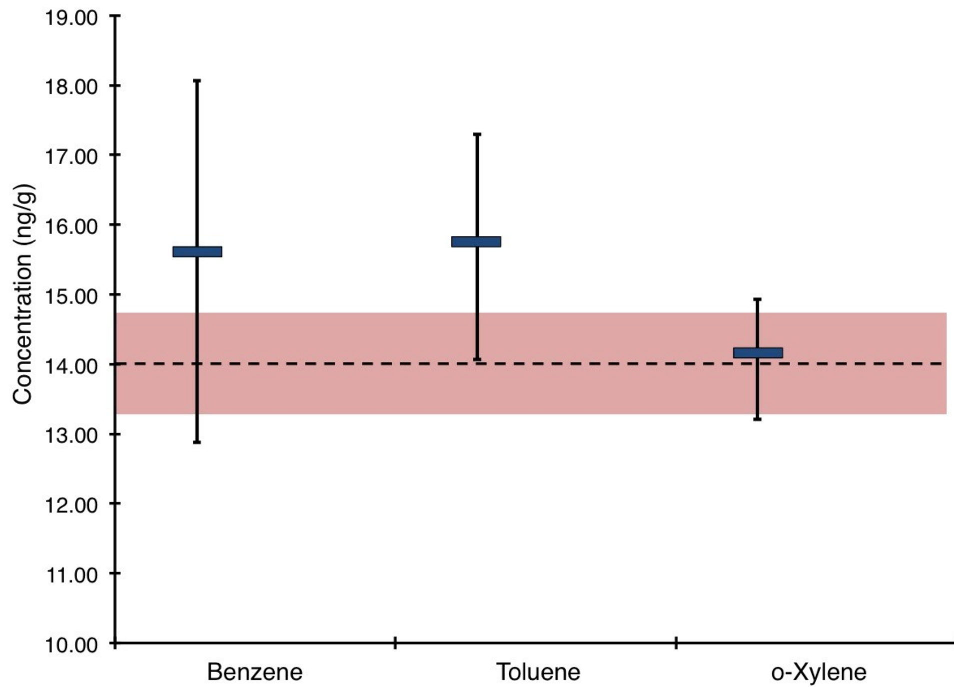


Figure 2.6: A comparison between the experimentally determined concentrations of all target analytes to be used in a collaborative clinical study with certified standards spiked into blank-subtracted serum for (top) PCB and (bottom) VOC compounds. Bars show 95% confidence of experimentally determined values and shaded area represents 95% confidence interval of certified standard.

The trace-level quantification of this research demanded the elimination of all potential background contamination to produce high-quality quantitative data. Sample analysis and lengthy automated sample sequence times may have allowed laboratory-air to contaminate the analytical process by a number of contaminant introduction routes: adsorption of volatile compounds from the laboratory air onto the SPME fiber as it sat unused, introduction of laboratory air into the GC system upon SPME injection into the inlet, or through an unknown introduction route. Background contamination was successfully reduced to levels below the limit of detection of the instrumental system by installation of a filter system containing activated carbon and a HEPA filter. Figure 2.7 demonstrates the improvement in background contamination that was observed when the enclosure was added to the GC/MS system. For determination of the effectiveness of the filter system, a high electron multiplier amplification was used, gain-factor of 9.0 (equivalent to an electron multiplier voltage of 2400 eV.)

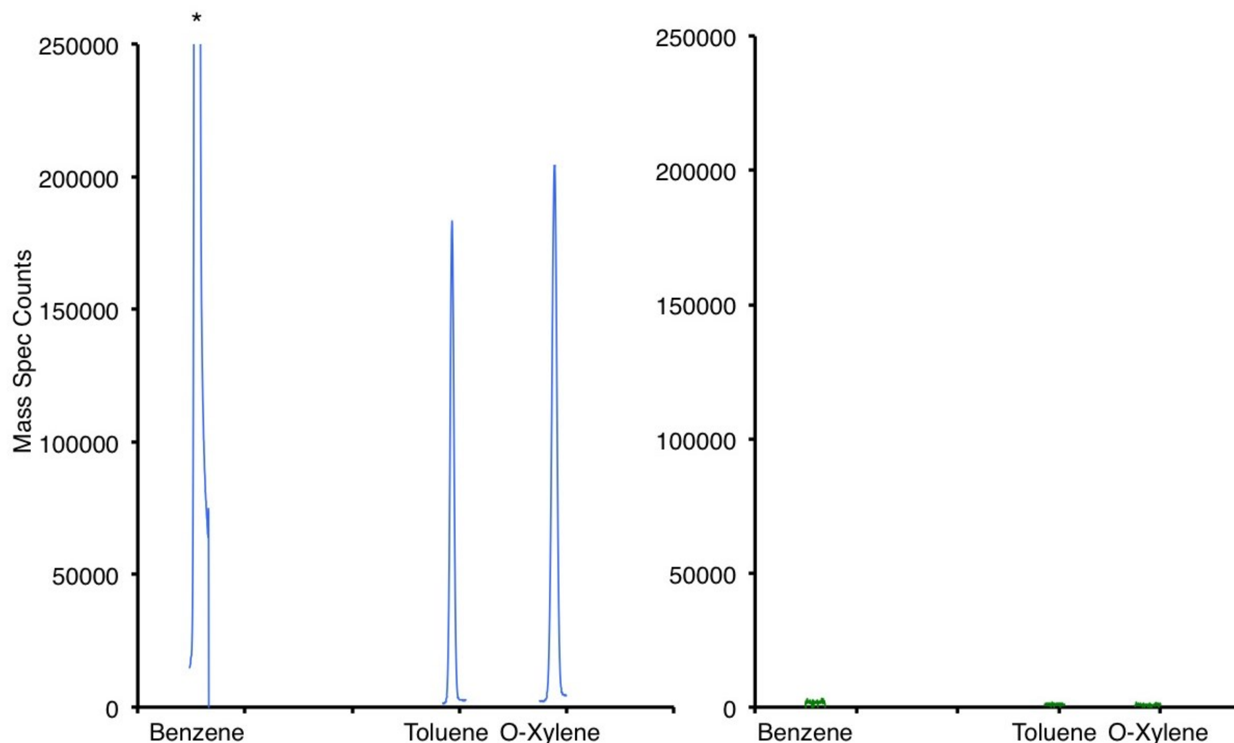


Figure 2.7: (left) Extracted ion chromatogram of the three most abundant contaminant peaks observed with a high electron multiplier amplification, and (right) the same extracted ion chromatogram showing baseline at the retention times associated with the contaminants
*Chromatographic peak extends above the limit of the y-axis

2.4 Application to Cleanroom Research and Autism Spectrum Disorders: A Pilot Study

2.4.1 Introduction to Clinical Study

Many researchers believe that the increase in autism diagnoses can be, at least in part, attributed to exposure to environmental pollutants.[22-24] While genetic factors clearly play a role in the development and maintenance of autism disorders,[25, 26] studies have provided indirect evidence to correlate autism diagnosis with exposure to a variety of environmental triggers like organophosphate pesticides,[27] as well as maternal exposure to traffic-related air pollution,[28] and thalidomide.[29] Further research is required to investigate, not only correlative relationship, but potential mechanistic relationships that may exist between environmental triggers and the

development of ASDs.[30] While there is no universal agreement on which, if any, environmental triggers play a role in the development and maintenance of autism disorders, nearly unanimous agreement exists among medical researchers that no evidence, direct or indirect, exists to correlate or link autism diagnosis with vaccinations.[31-34]

Multiple studies have demonstrated the importance of early treatment interventions in individuals with ASDs. [35-37] It has been shown that younger children with autism display greater and more rapid response to intensive treatments, and that there may be a crucial time interval for intervention before the end of early life developmental plasticity. [35] It was therefore hypothesized in a collaborative pilot study with The Children's Institute of Pittsburgh that an intervention that temporarily decreases environmental exposure to an individual, by isolation in a cleanroom environment, may reduce the presentation of autism symptoms. Results of this work could allow researchers to evaluate the feasibility and potential effectiveness of a full-scale study.

2.4.2 VOC and PCB Mean Quantitative Results

Comparing mean concentrations of each analyte across the ten participants before a cleanroom intervention to mean concentrations after yielded no statistically significant change. Table 2.4 demonstrates that all participants displayed quantifiable concentrations of benzene, toluene, o-xylene, and PCB-101. Before and after concentration changes for toluene and PCB-28 demonstrated the highest statistical significance ($p=0.0769$ and $p=0.0829$, respectively), but did not meet the cut-off of $p<0.05$. Mean quantitative results for all analytes pooled across the entire study before and after cleanroom intervention can be found in table 2.4. Low precision obtained in the quantification of VOC and PCB compounds was a significant limitation in statistical comparison of pre- and post-cleanroom mean concentrations.

Table 2.4: Mean concentration (ng/g serum) of all studied analytes in all ten children both before and after the cleanroom, showing 95% confidence and the significance of before/after difference

	Before Cleanroom			After Cleanroom			
Analyte	Concentration	95% CI	n	Concentration	95% CI	n	p-value*
Benzene	9.49	3.8	10	7.52	3.6	10	0.253
Toluene	0.747	0.20	10	0.950	0.27	10	0.0769
o-Xylene	0.8259	0.12	10	0.867	0.19	10	0.56
PCB-28	0.519	0.16	6	0.721	0.21	7	0.0829
PCB-52	0.688	0.22	8	0.613	0.18	8	0.469
PCB-101	6.18	2.1	10	7.23	2.3	10	0.305
PCB-138	0.695	0.20	6	0.661	0.14	6	0.737

*P-value comparison between mean concentration of each analyte before and after cleanroom

n: Number of children displaying quantifiable concentrations of specified analyte

2.4.3 Age-Dependent Detoxification Trends

Analysis of mean concentrations found that the change in serum benzene concentration negatively correlated with age with high statistical significance and high negative correlation ($p=0.021$, $r= -0.71$). There were no significant differences in VOC concentrations in paired t-tests of pre- and post-cleanroom findings for the set of ten children. However, this calculation did not account for the high imprecision present in the quantification of benzene.

Analysis of mean concentrations found that the change in serum PCB-28 negatively correlated with age with high statistical significance and high negative correlation ($p=0.028$, $r= -0.72$), as seen in figure 2.8. PCBs 52, 101, and 138 were not found to have undergone significant changes in concentration between pre- and post-cleanroom measurements. PCBs 31, 153, and 180 were not quantifiable in any of the ten children. There were no significant differences in PCB concentrations in paired t-tests of pre- and post- findings for the entire cohort. However, as with benzene, the high degree of uncertainty present in the quantification of PCB-28 eliminates the drawing of conclusive, medically relevant results.

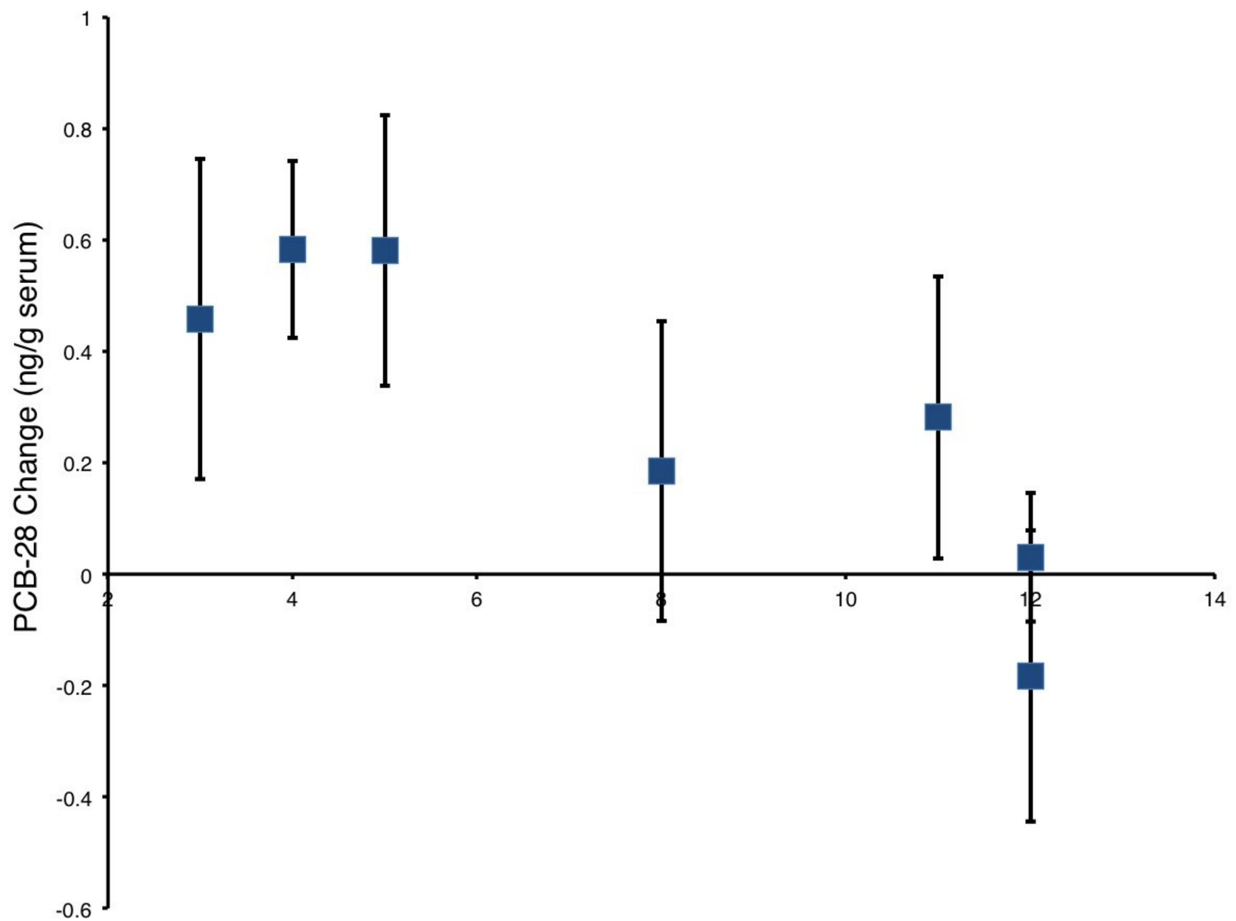


Figure 2.8: Figure 2.4: The change in PCB-28 serum-concentrations between pre-cleanroom and post-cleanroom measurement, arranged by age of child. Dashed line represents no change. $p=0.028$, $r=-0.72$

The increase in the means of benzene and PCB-28 serum-concentrations after the cleanroom in the younger children correlated with an improvement on the autism rating scales and observations.[16] However, due to confounding variables, such as the pilot-nature of this study, lack of a control cohort, and high degrees of uncertainty present in quantitative results, the observed neurological improvements could not be unequivocally attributed to an improvement in autism severity. The results of this study indicate that a full-scale investigation of the effects of a cleanroom environment on the severity of autism symptoms in children with ASDs and

neurotypical controls is a reasonable next step. Future research in full-scale implementation should include a controlled cohort-study with a increased sample size using a 24 hour per day cleanroom with control over participant diet. Additionally, improved analytical methods must be developed to increase quantitative quality and decrease uncertainty.

2.5 Conclusions and Preliminary Findings

A method for the analysis of VOC and PCB compounds in microliter volumes of human serum was modified from literature sources by the addition of IDMS quantification. The method was optimized for the specific analytes of interest and validated against matrix-matched certified standards. This method was the first to specifically correct for the irreproducible nature of SPME by the addition of true IDMS quantification. The optimized and validated method demonstrated high accuracy and precision, as well as a sensitivity to be useful for environmentally relevant quantification.

The validated analytical method was applied to a collaborative pilot study assessing the effect of a cleanroom sleeping environment on the xenobiotic body-burden of children with autism. The developed and optimized method provided high analytical quality in method development and validation using blank-subtracted blood serum spiked with analytes of interest under strictly controlled sample preparation parameters. Greater control must be gained over the entire sample analysis process to ensure that highly precise data are obtained from real clinical samples. While the feasibility of such a study was demonstrated, improved analytical methods must be developed prior to implementation of full-scale research.[16]

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Chapter 3: Simultaneous extraction of volatile, semi-volatile, and non-volatile compounds from environmental waters and biological samples

3.1 Introduction

The rapidly developing environmental human health field of exposomics requires specialized and optimized analytical methods to be accurately and reliably deployed academic, government, clinical, environmental, and commercial laboratories. As the emergent field advances, study of the human health impact of environmental pollutants has increasingly focused on chronic exposure to POPs (e.g. long-term ingestion and inhalation from environmental and industrial sources). Thus, regional and demographic data concerning human exposure to specific pollutants has become increasingly important to medical and academic researchers.[1]

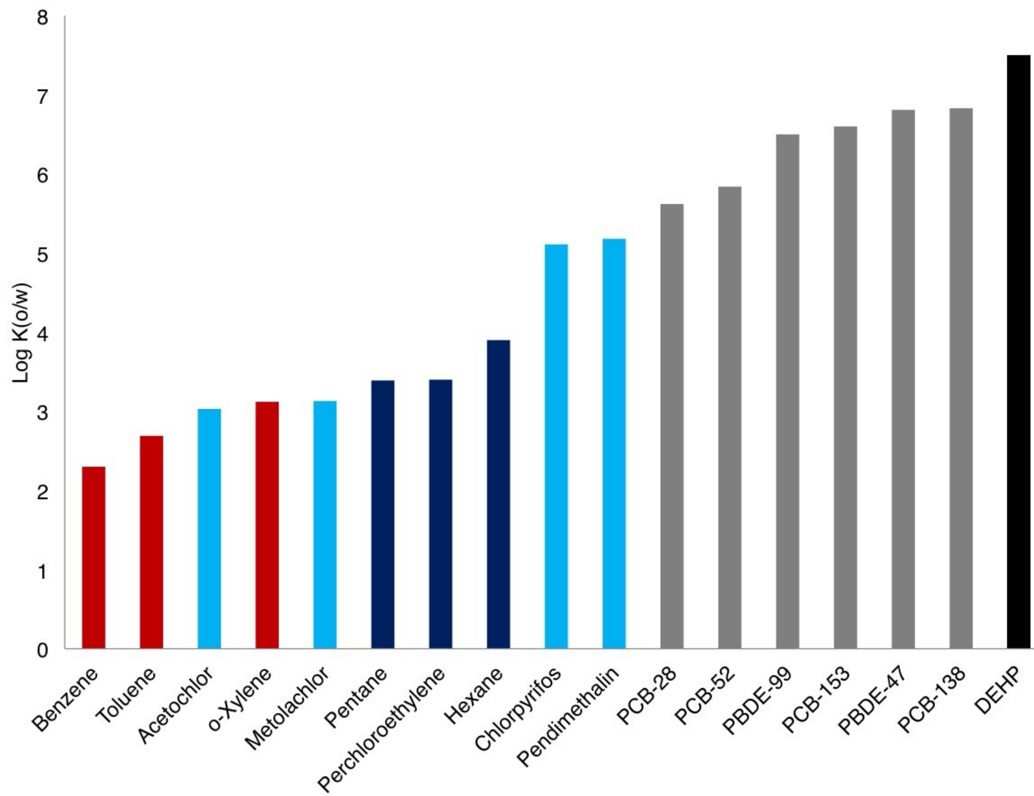
Compound selection for this method development work was based upon previous research implicating specific compounds and chemical classes relevant for exposomic and environmental human health research. The compounds chosen for this development work can be found in table 3.1.

Table 3.1: Compound list for method development experiments

Benzene	PCB-28
Toluene	PCB-52
o-Xylene	PCB-101
Chlorpyrifos	PCB-138
Pendimethalin	PCB-153
Metolachlor	PBDE-47
Acetochlor	PBDE-99
DEHP	

Additionally, compounds chosen for method development represented a significant range of volatility and hydrophobicity (measured as the octanol/water partition coefficient, $K_{o/w}$).

Figure 3.1a and figure 3.1b demonstrate the several orders of magnitude of volatility (kPa) and hydrophobicity ($K_{o/w}$) of the target compounds used this this research. The purpose of including such varied compounds was to demonstrate the potential applicability of a developed method to a significantly wide range of future analytes of interest- enabling future expansion of a developed method to additional compounds of interest that may fall within the demonstrated range of chemical characteristics.



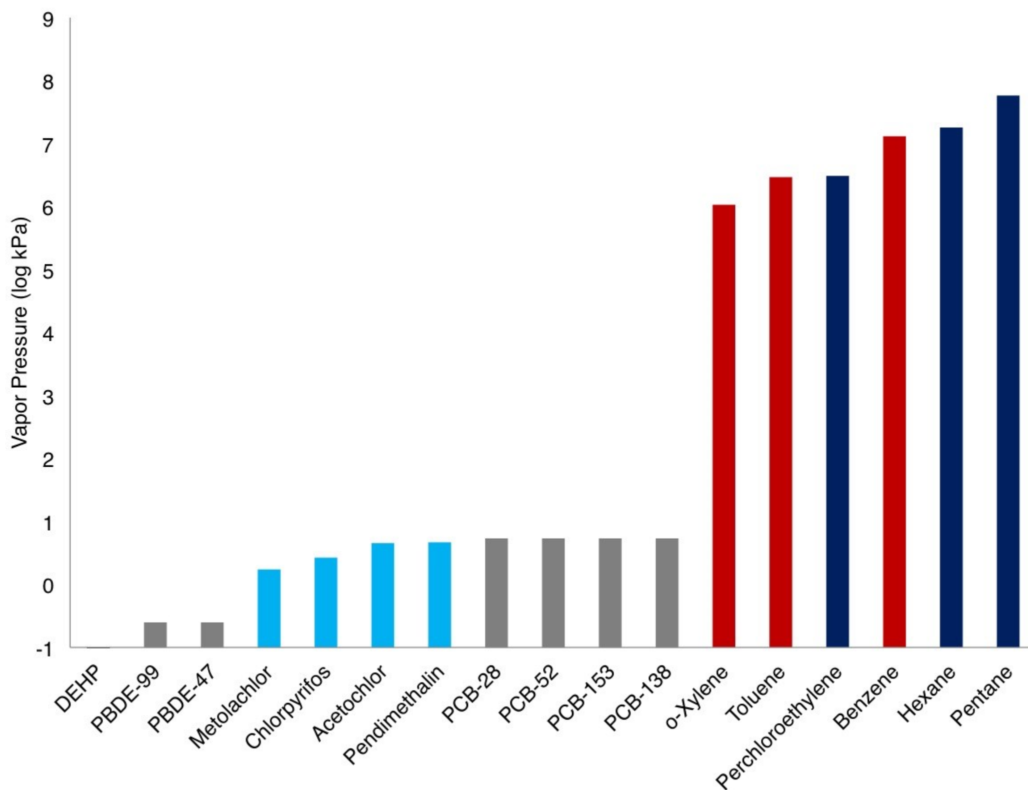


Figure 3.1: Demonstration of the wide range of compounds encapsulated in this method development work, showing hydrophobicity (top) and volatility (bottom).

3.2 Background

The assessment of the interaction between the environment and human genetics and epigenetics is called exposomic research. This section describes a method that was developed for the extraction and quantification of select POPs in human serum, to build upon the SPME method described in Chapter 2. This developed method was then applied to assessed in an equally complex matrix: drinking water and wastewater analysis, with the purpose of updating outdated standard Methods.

As described in Chapter 2, SPME proved to be useful for only headspace analysis, contrary to published researched. Thus a new method that accomplished all major POPs categories volatile, semi-volatile and non-volatile had to be developed. This chapter discusses this development and the attributes that permitted the RBC to be accomplished as later discussed.

It has been shown that desorption of analytes from the stir bar sorbent phase prior to analyte-extraction phase equilibrium yields non-reproducible results.[2] However, driving these extractions to equilibrium often requires prohibitively long extraction times; studies have shown that up to 14 hours is often required for complete equilibration of POPs compounds between the extraction phase (PDMS) and the solution.[3, 4] Citing equilibration time constraints, studies have indicated the need to develop techniques to ensure pre-equilibrium sample-to-sample reproducibility in SBSE.[5] A direct mathematic quantification eliminates inaccuracy introduced by relative quantification methods like calibration curve and response-factor (RF) correction.[6] Accurate quantification is accomplished by isotope dilution mass spectrometry, as described by EPA Method 6800.[7] EPA and peer reviewed methods using isotope dilution quantification for POPs require the analyst to generate calibration curves based upon relative isotopic RFs. All forms of calibration are relative by nature. True isotope dilution is a direct quantification that

avoids the relative nature of external calibration curves or RFs. This type of quantification is called isotope dilution mass spectrometry (IDMS).

Environmental sources of human exposure to POPs can include air, foods, manufacturer-treated products in the home, and municipal water supply, among many others. The analysis of differing contaminated matrices have historically required varying analytical procedures for extraction and quantification of POPs. Water, specifically drinking water and wastewater, are carefully regulated by the US EPA and the Clean Water Act (CWA). The EPA has produced standard methods for the analysis of a variety of POPs in multiple aqueous matrices. In total, 18 EPA methods have been produced by the standards outlined in the CWA to govern the quantification and/or identification of organic pollutants in aqueous matrices for public health. The most comprehensive of these methods is EPA Method 625, which specifically outlines a procedure for the quantification of 47 POPs from municipal water and industrial wastewater. Developed in the early 1980s, Method 625 requires liquid-liquid extraction as a means of sample preparation and isolation. This type of extraction uses large volumes of toxic solvents to extract hydrophobic compounds from aqueous solutions. In the 1990s, a separate EPA Method was developed, Method 525.2, for the analysis of a similar suite of POPs in drinking water. Method 525.2 improved upon Method 625 by incorporating the use of solid-phase extraction techniques to eliminate liquid-liquid extractions.

One of the primary objectives of this research was to increase efficiency and reduce variance in sample preparation, while simultaneously improving quantitative accuracy without using external calibration curves or isotopic RFs. To increase efficiency, this research optimized single stir-bar SBSE to extract both volatile and nonvolatile analytes from a sample in a single extraction. To improve SBSE reproducibility and accuracy, IDMS was applied to reduce the

influence of pre-extraction equilibrium analysis and imprecise sample preparation on quantitative quality. For the purpose of clinical application, sample volume was optimized to significantly reduce the volume required compared with existing methods. Another objective of this work was to validate SBSE for use with EPA Method 625 as an improved sample preparation technique. Application of the developed method to both clinical and environmental samples was validated using certified standards. The transferability of the automated method was assessed using an independent laboratory and separate analyst.

3.3 Materials and Methods

3.3.1 Reagents and Materials

For quantification in blood serum, the following standards were acquired: a standard containing 7 polychlorobiphenyl (PCB) congeners [2,4,4'-PCB (PCB-28), 2,2',5,5'-PCB (PCB-52), 2,2',4,5,5'-PCB (PCB-101), 2,2',3,4,4',5-PCB (PCB-138), 2,2',4,4',5,5'-PCB (PCB-153), 2,2',3,4,4',5,5'-PCB (PCB-180), and 2,2',3,3',4,4',5,5'-PCB (PCB-209)] and standards for polybrominated diphenyl ether (PBDE) congeners [2,2',4,4'-BDE (PBDE-47) and 2,2',4,4',5-BDE (PBDE-99)] were purchased from Sigma-Aldrich (St. Louis, MO). Benzene, toluene, *o*-xylene, (≥99% purity) and bis(2-ethylhexyl) phthalate (DEHP) were purchased from Fluka (St. Louis, MO). Certified standards for chlorpyrifos (99.5% pure), pendimethalin (98.8% pure), acetochlor (98% pure), metolachlor (98.6% pure), toluene-d_8, *o*-xylene-d_{10} were obtained from SPEX CertiPrep Group (Metuchen, NJ). A certified standard for benzene-d_6 was obtained from Cerilliant (Reston, VA). Chlorpyrifos-d_{10} (99% labeled), a standard mixture of PCB-$^{13}C_{12}$ (99% labeled, and standards for PBDE-47 and 99-$^{13}C_{12}$ (99% labeled) were obtained from Cambridge Isotope Laboratories, Incorporated. (Tewksbury, MA). Pendimethalin-d_5 (98% labeled),

acetochlor-d_{11} (98% labeled), metolachlor-d_6 (98% labeled), and bis(2-ethylhexyl) phthalate-d_{38} (DEHP-d_{38}) were obtained from C/D/N Isotopes Incorporated. (Pointe-Claire, Quebec, Canada).

For quantification of POPs in drinking and waste waters, the following standards were acquired: a mixture of a mixture of 4-bromophenyl phenyl ether-d_5, 2-chloronaphthalene-d_7, 4-chlorophenyl phenyl ether-d_5, di-n-butyl phthalate-d_4, diethyl phthalate-d_4, di-n-octyl phthalate-d_4, hexachlorobenzene-$^{13}C_6$, hexachloroethane-^{13}C, isophorone-d_3, trichlorobenzene-d_3, at 98% label purity and 99% chemical purity at 5000 μg/mL; a mixture of acenaphthene-d_{10}, anthracene-d_{10}, benzo[k]fluoranthene-d_{12}, bis(2-chloroethyl) ether-d_8, chrysene-d_{12}, fluorene-d_{10}, naphthalene-d_8, pyrene-d_{10}, at 98% label purity and 99% chemical purity at 2500 μg/mL; and 0.01g of β-BHC-$^{13}C_6$, 4,4'-DDT-d_4, and 4,4'-DDD-d_4. Unlabeled analogs of each POP were acquired at 99% chemical purity for method development, optimization, and validation.

Extractions were carried out using 10 mm x 0.5 mm (length x film thickness) polydimethyl siloxane (PDMS) stir-bars, supplied by GERSTEL (Mülheim a/d Ruhr, Germany) in 10 mL headspace vials for extractions (Sigma-Aldrich). Stirring was conducted using a 20-position magnetic stir-plate (GERSTEL). A SPME assembly was purchased with a PDMS fiber of 30 μm film thickness (Sigma-Aldrich) for method comparison studies. Prior to use, the stir-bars were conditioned per manufacturer instructions. Thermal conditioning took place in a thermal conditioning unit (TCU) at 290 °C for 4 hours with a helium flow of 50 mL/min. HPLC-grade methanol (Sigma-Aldrich) and ultrapure (18 Ω) water were used for reagent dilution, glassware cleaning, and stir-bar cleaning.

3.2.2 Analyte Selection

For development and optimization, previous studies guided the choices of the most representative analytes to encompass the many classes of chemical activity defined as POPs. For biological analysis, traditional POPs classes were included (PCB, PBDE), along with compounds that have not traditionally been classified as POPs but display the characteristics of semi-volatility, environmental persistence, and hydrophobicity.[8] Many of the analytes chosen for this research are not present on the Stockholm Convention list of POPs; this work was designed to include both banned POPs and products with similar chemical characteristics which have become environmentally ubiquitous.[9, 10] Selected compounds ranged several orders of magnitude in hydrophobicity (octanol:water partitioning coefficient, $K_{o/w}$) and volatility (torr). Three volatile organic compounds (benzene, toluene, and *o*-xylene), five PCBs (standard congeners 28, 52, 101, 138, and 153), two PBDEs (standard congeners 47 and 99), two organochlorine pesticides (metolachlor and acetochlor), one dinitroanaline pesticide (pendimethalin), one organophosphate pesticide (chlorpyrifos), and one phthalate (bis(2-ethylhexyl) phthalate) were chosen for this research.

For drinking water and wastewater, an additional 19 compounds were chosen from the list of base-neutral compounds listed in EPA Method 625, representing a several order of magnitude range of $K_{o/w}$ and volatility. The complete compound list found in EPA Method 625 was reduced to 19 to include the complete breadth of volatility and hydrophobicity found in Method 625, while eliminating compounds of similar chemical characteristics.

3.3.3 Sample Processing

For preparation of blood serum: in an International Standards Organization Class 5 cleanroom, whole blood samples obtained in an internal review board-approved collaboration with The Children's Institute of Pittsburgh (Pittsburgh, PA) were separated into red blood cells and serum using a calibrated centrifuge. Both serum and red blood cells were immediately transferred to airtight polyethylene containers and stored in the dark at -80 °C.

For preparation of industrial wastewater: synthetic wastewater samples were prepared according to ASTM-D5905 procedures. Stock wastewater was divided into two solutions: synthetic wastewater and synthetic wastewater modified with 20% acetonitrile.

3.3.4 Stir-bar Extractions

For development and validation work on blood serum, samples were brought to room temperature and approximately 200 μL was added by mass to a 10 mL extraction vial using an analytical balance. A mixture of enriched isotopic analogs was prepared by mass at a concentration equal to the certified concentration of the target analyte and spiked by mass into each sample vial. For this study, 100 μL of a mixture composed of ~25 μg/g benzene, toluene, *o*-xylene; ~4 μg/g PCB-28, PCB-52, PCB-101, PCB-138, PCB-153; ~5 μg/g PBDE-47, PBDE-99, chlorpyrifos, metolachlor, acetochlor, pendimethalin, and DEHP was accurately spiked into each vial of serum.

For work on drinking water and wastewater, 9.5 mL of stock wastewater (either unmodified or acetonitrile modified) were added to a 10 mL sample vial. A mixture of enriched isotopic analogs of each EPA 625 analyte to give a final approximate concentration of 100 ng/g of each enriched compound. All blanks and samples were prepared following EPA Method 625

and the internal document "ITN-261: Standard and Sample Preparation for EPA 625 Study," where it amended EPA Method 625. This method was modified for use with IDMS in minor, but significant, ways. A second analyte mixture was prepared that contained an isotopically mass-upshifted labeled analog for each analyte included in this study. The isotopic analogs in the mixture were at accurately known concentrations, such that each labeled molecule could be spiked into a wastewater sample within one order of magnitude in concentration of its unlabeled counterpart.

Following the standard method, each sample replicate was analyzed with two stir bars: one extracting from an unmodified solution and one extracting from a 20% acetonitrile solution. Each sub-sample and blank was prepared following the standard method; however, prior to stir bar extraction, 1.6 mL of the mixture of labeled analytes was spiked by mass into each sub-sample vial. Labeled-unlabeled equilibration occurred during the stir-bar extraction process, which was conducted for 60 minutes.

For dual SBSE, one bar was added to the vial with 2 mL of ultra-pure water and one bar was hung in the headspace on a metallic wire with string. For S-SBSE, one stir-bar was immersed in the solution and ultra-pure water was added such that the extraction vial was completely filled to eliminate as much headspace as possible. For blood serum, methanol was added, before extraction, to each sample to achieve a final methanol concentration of 20% (including the methanol from the isotope mixture) after dilution. For water samples, no solvent was added during sample preparation- as acetonitrile was added in the stock dilution step. Teflon-lined screw caps were fixed to the extraction vessels. Extraction was conducted at 1500 rpm for 60 minutes. The stir-bars were removed from the sample with tweezers and thoroughly

rinsed with ultrapure water and dried with a lint-free tissue and deposited into a glass thermal desorption tube. Dual SBSE and single SBSE are compared graphically in figure 3.2.

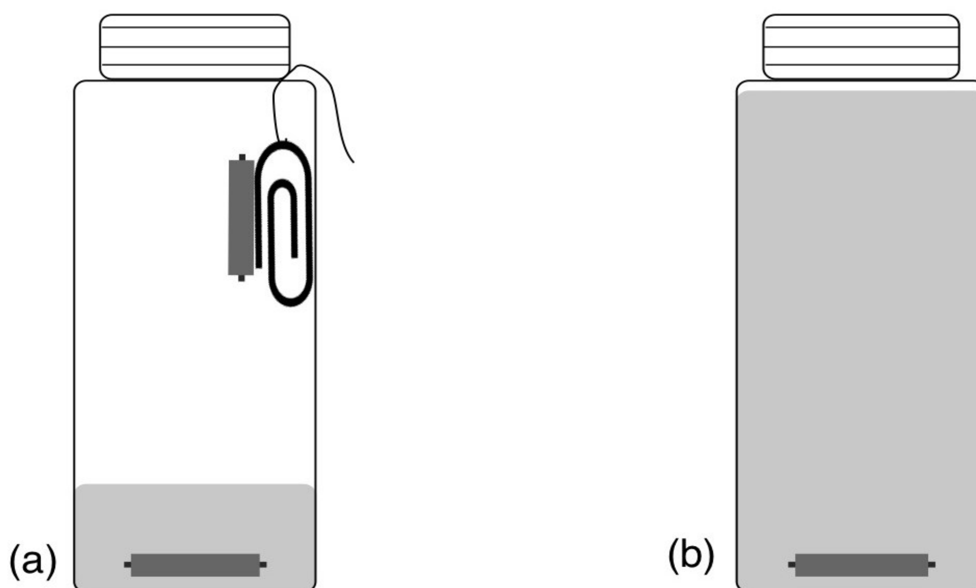


Figure 3.2: Schematic of a two stir-bar extraction method in which volatile and semi-volatile compounds are extracted (a) from the headspace on one stir-bar and the nonvolatile compounds on a second immersed stir-bar or (b) simultaneously with the nonvolatile compounds on one stir-bar immersed in the diluted solution.

3.3.5 Desorption and Chromatography

Desorption tubes were loaded into a tray and introduced sequentially into the thermal desorption unit (TDU) (GERSTEL). The GC inlet was set to use programmed-temperature vaporization (PTV) on a chilled injector system (CIS-6) (GERSTEL) inlet containing a CIS/TDU inlet liner packed with Tenax TA^{TM} (Buchem B.V., Apeldoorn, The Netherlands). The CIS-6 injector was installed in an Agilent 6890 GC - 5975 MS system (Agilent Technologies, Santa Clara, CA, USA). The sample loading and handling was performed by a dual-head robotic multipurpose sampling system (MPS-2) (GERSTEL). The method parameters were programmed to the final desorption temperature of 280 ºC and the analytes were desorbed under helium in the

TDU before cryofocusing at -70 ºC in the PTV system with liquid nitrogen. Finally, the CIS system was ballistically heated at 720 ºC/minute to 280 ºC to transfer the analytes to the GC-MS for analysis. The analytes were separated chromatographically using an HP-5 MS column (30 mx0.25 mm I.D. 0.25 µm film thickness, 5%-phenyl polydimethylsiloxane) at a 1.0 mL/minute carrier gas flow rate. The GC oven was heated from 45 ºC to 280 ºC at 12 ºC per minute, where it was held for 15 minutes. Ionization was conducted in electron ionization mode and mass selection / detection was accomplished in select ion mode (SIM) programmed to the quantitative and secondary ions selected for each analyte in the method development stage.

3.3.6 Inter-Laboratory Analytical Method Transfer

Unenriched versions of one organochlorine pesticide, one dinitroanaline pesticide, one tetra-substituted PCB, two co-eluting hexa-substituted PCBs, and one PBDE were spiked into a mixture of ultrapure water. Five replicates were processed at an off-site, independent laboratory by an analyst that received minimal training (i.e. two hours) on the extraction, analysis, and quantification steps. Accompanying the reagents and supplies were electronic versions of the analytical protocol, instrumental and data processing methods, and an automated internally developed quantification software. Samples were processed by the independent analyst and final quantitative values were generated automatically and sent back to the primary laboratory for comparison.

3.4 Blood Serum Results and Discussion

A method was developed, optimized, validated, and explored for clinical application using blood serum and organic environmental pollutants related to human-health measurements. This section will describe the results of these experiments as they relate to analysis of blood serum only. The analytical method was optimized to produce a resolved chromatographic spectra containing all analytes of interest. Figure 3.3 demonstrates the fully resolved chromatogram containing all analytes of interest.

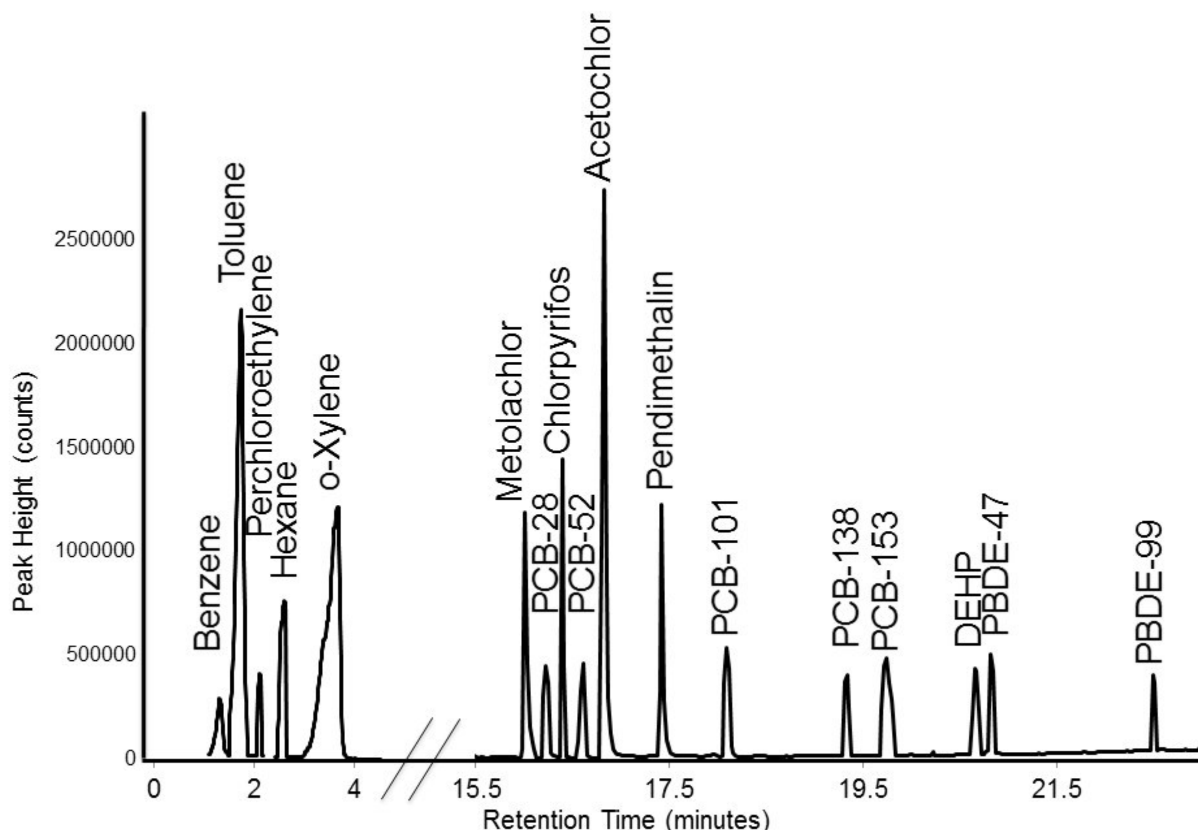


Figure 3.3: Resolved chromatogram generated using the optimized method developed in this work, showing all analytes of interest

3.4.1 Single vs. Dual Stir-Bar Extraction

Single and dual bar extraction recoveries were compared by replicate extractions of a prepared mixture of endogenous NIST-traceable standards by each method. Total analyte recovery with single bar SBSE was comparable with dual bar SBSE, producing statistically identical recovery for 13 out of 15 analytes. As figure 3.4 demonstrates, the highest volatility analytes, benzene and toluene, presented recoveries 85% and 45% lower, respectively, in single step extraction compared with dual SBSE. Other POPs typically extracted using headspace methods: PCBs, PBDEs, phthalates, and pesticides displayed statistically identical recoveries using the single step analysis.

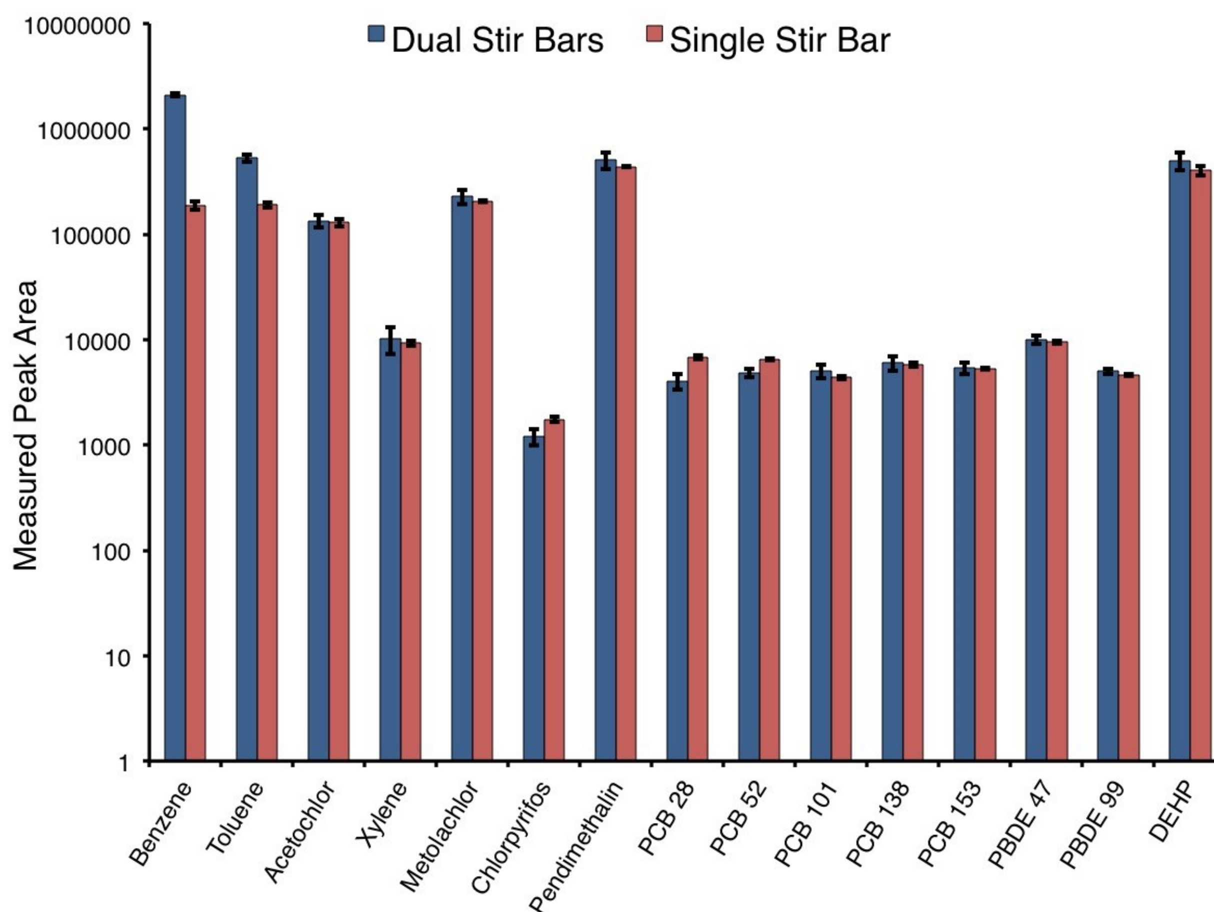


Figure 3.4: A comparison of relative recovery between single and dual stir bar extraction, showing 95% CI (n=5)

Single bar SBSE increased method efficiency and allowed twice as many samples to be extracted simultaneously, while producing recoveries comparable to dual SBSE. Sorbent stir bars possess an inherent lifespan. Manufacturer recommendations list this lifespan at 50-extractions; however, internal research determined that under light use (moderate pH and primarily aqueous samples), nearly 100 extraction could be performed before extraction efficiency fell below acceptable levels. The increasing popularity of SBSE stir bars, combined with their relatively high cost, demands that laboratories make the most efficient use of the available number of bars. The improved efficiency afforded by extracting with one stir bar instead of two would most obviously benefit laboratories that process large numbers of mixed-volatility samples.

3.4.2 Sample-to-Sample Reproducibility

Replicates of a prepared mixture of NIST-traceable endogenous standards were extracted from blood serum by single bar SBSE to determine sample-to-sample reproducibility. Prior to quantification, analysis was performed on the reagent serum to determine the concentration of any existing background contamination present in the serum. These background values were subtracted from any data obtained from the reagent serum, producing a so-called "blank-subtracted serum." Standards were added to the serum using an analytical balance, to enable quantification by mass, at concentrations within one order of magnitude of the respective LOQ for each analyte. Isotopic forms of each compound were spiked into each sample vial by mass. Precision was assessed as %RSD for comparative purposes. Unaltered peak areas were used to obtain "raw data" %RSD. This same data was then processed as a simple function of endogenous to spiked peak areas ($P_{endogenous}/P_{spike}$). As seen in table 3.2, and represented graphically in figure

3.5, analyzing by raw peak areas produced mean total %RSD across all analytes of 11.9%. When the same data were processed by IDMS, mean total %RSD for all analytes improved to 4.2%.

Table 3.2: A comparison of reproducibility of raw chromatographic peak areas with IDMS-corrected peak areas (n=5)

Analyte	%RSD Raw Data	%RSD IDMS Corrected
Benzene	30	9. 4
Toluene	17	5.3
o-Xylene	14	5.7
PCB-28	14	4.5
PCB-52	11	2.3
PCB-101	7.3	3.6
PCB-138	9.2	4.1
PCB-153	11	2.0
PBDE-47	3.3	3.0
PBDE-99	6.0	1.4
Chlorpyrifos	9.8	5.7
Metolachlor	7.0	2.0
Acetochlor	19	8.5
Pendimethalin	10.1	0.812
DEHP	10.8	6.12

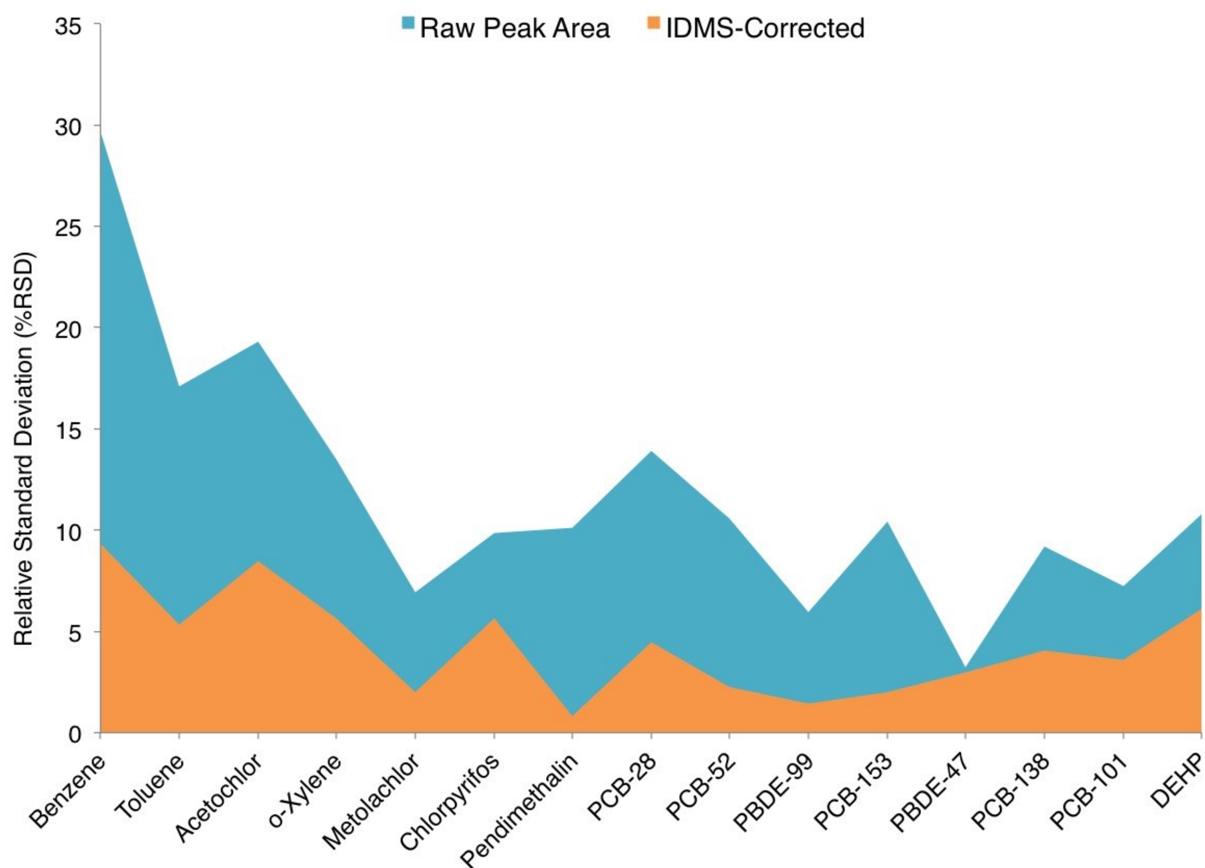


Figure 3.5: The comparison between relative standard deviation (%RSD, n=5) of all compounds of interest quantified in blank-subtracted serum. Background represented the %RSD of raw peak areas of all replicates, foreground represents %RSD of IDMS-corrected peak area

Significant improvements in sample-to-sample reproducibility observed in the IDMS data were most reasonably achieved by compensating for analytical variance typically introduced to the sample preparation and quantification. Many peer reviewed and EPA methods still in use today define isotope dilution as an isotopic RF. Isotopic RF quantification relies on generating a calibration curve that plots RF (between an endogenous compound and its isotopic analog) versus concentration of endogenous standard.[11] This approach differs from IDMS, in which no calibration curves or RFs are generated. This way, IDMS eliminates the inexact nature of calibration curves. By decreasing variance, IDMS quantification created confidence intervals that

were narrower than those obtained by analysis of raw data, allowing for potentially actionable diagnostic results.

3.4.3 Comparison with Existing Methods

Single step extraction was compared with the traditionally accepted extraction method of SPME using both isotopic RF and IDMS mass spectrometry quantification of PCB-52 in 200 μL of blank-subtracted serum. Figure 3.6 shows that SPME coupled with isotopic RF quantification produced inaccurate quantitative values with poor reproducibility. The addition of IDMS quantification to SPME improved accuracy from 24.6% to 9.66% error and precision from 16.9% to 5.61% within-run RSD. Single bar SBSE improved the accuracy over SPME from 9.66% to 6.07% error and increased the precision from 5.61% to 5.40% RSD. Single bar SBSE-IDMSMS significantly improved quantitative accuracy and precision, compared with industry-accepted SPME and calibration curves. A reasonable explanation for the improvements in SPME-IDMS over SPME-RF is the ability for IDMS to reduce or eliminate many errors associated with sample preparation and analysis once isotopically labeled analog and naturally occurring analyte are in equilibrium.

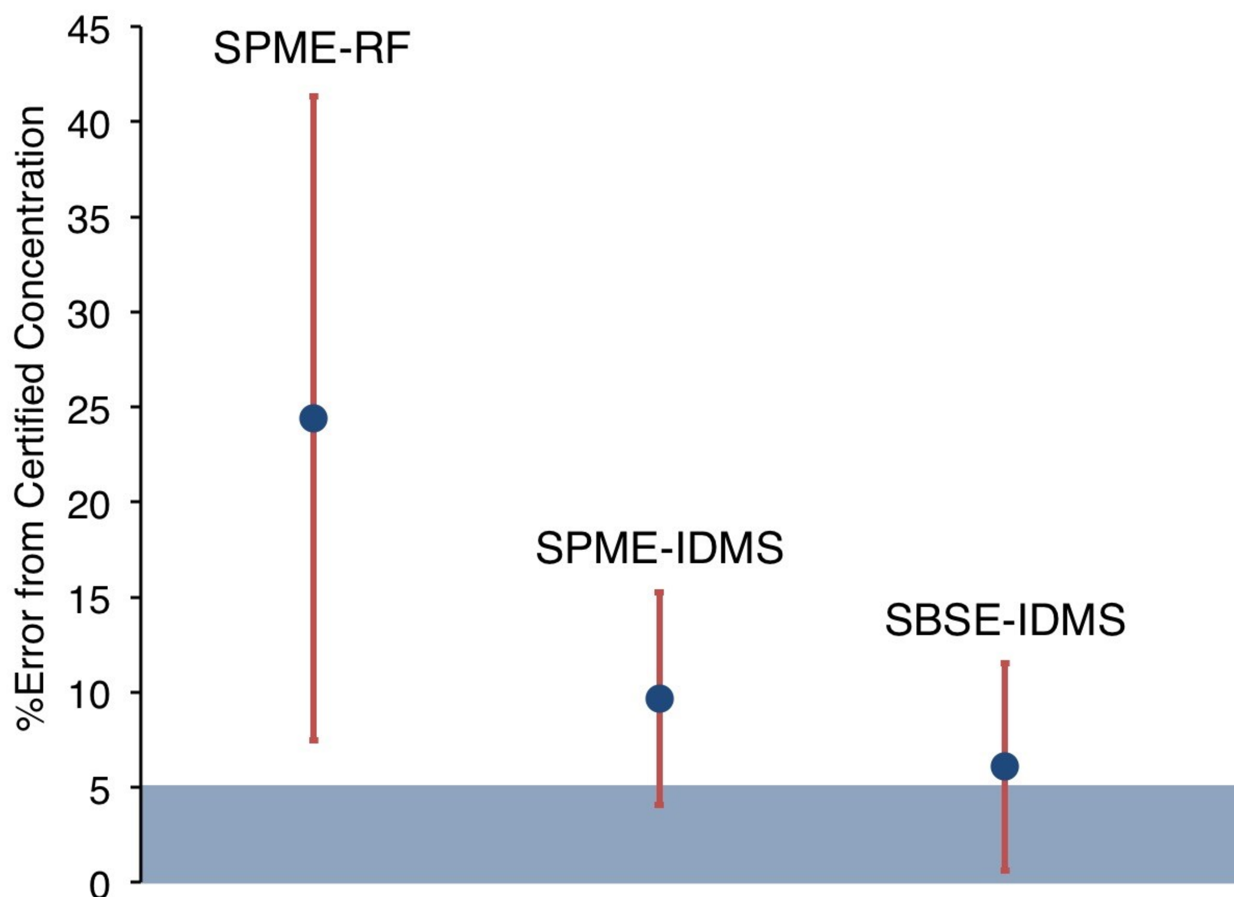


Figure 3.6: A comparison of single bar SBSE and IDMS with existing, industry-accepted methods of extraction and isotopic quantification. The 95% CI (n=5) is shown. Uncertainty of the calculated concentration is shaded.

3.4.4 Accuracy at Limit of Quantification

Data quality approaching a limit of quantification (LOQ) was assessed for both RF and IDMS quantification using PCB-52 and a quantification limit of 0.111 ng/g. Data was obtained by spiking isotopic and varying concentrations of endogenous PCB-52 into 200 μL of blank-subtracted serum and analyzing by single bar SBSE-IDMS. An isotopic RF calibration curve was generated for the endogenous compound at values 40% to 4000% above LOQ with n=5 replicates at each point. This data was then processed by IDMS using data obtained from the same analyses. Figure 3.7 shows that the RF data lost quantitative accuracy below 25 ng/g (two

order of magnitude above the IDMS quantification limit) with a mean within-run %RSD of 10.6%. When the same data was processed by IDMS, all data points maintained quantitative accuracy with a mean within-run %RSD of 2.28%.

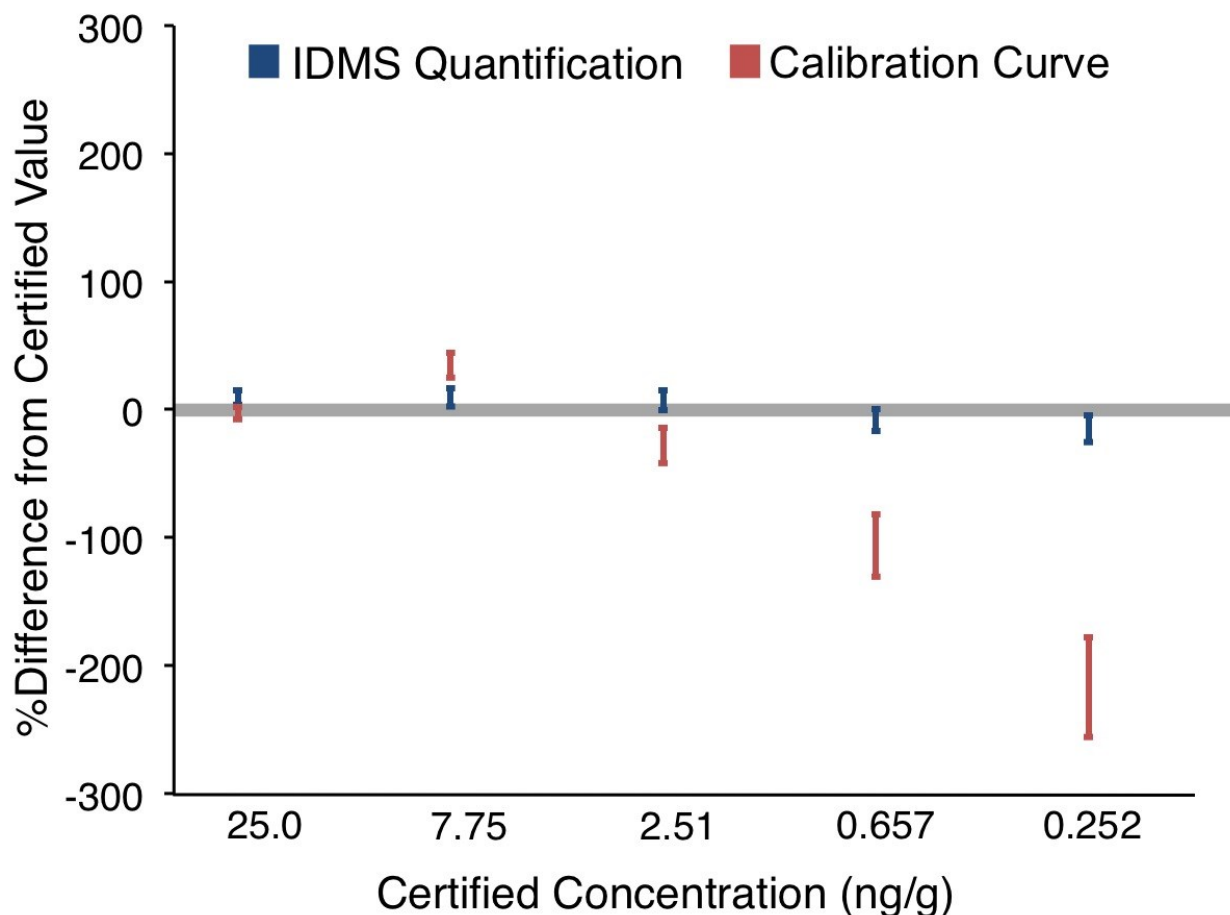


Figure 3.7 A comparison of IDMS and RF quantifications approaching the PCB-52 quantification limit, showing 95% CI (n=5). Uncertainty of the certified value (5%) is shaded.

As suggested by theory, it was observed that calibration accuracy and precision worsened approaching the limit of quantification.[12] The calibration curve does not match the certified concentration below 25.0 ng/g. This work demonstrated the capability of IDMS to maintain quantitative accuracy, validity, and reliability approaching the instrumental LOQ.

3.4.5 Method Validation

Concentrations of all analytes were experimentally determined in 200 μL of blank-subtracted serum and compared at the 95% CI against certified standards traceable to the National Institute of Standards and Technology (NIST). All quantified POPs were statistically identical to the NIST traceable certified concentrations, proving accurate quantification. A complete description of scientifically relevant chemical characteristics and figures of merit of the single bar SBSE-IDMS mass spectrometry method can be found in table 3.3. All data were obtained from analyses of samples containing endogenous and isotopic compounds spiked into blank-subtracted serum using an analytical balance. The calculated value represents the concentration of endogenous compounds present in the spiked serum prior to analysis calculated from the initial concentrations of NIST-traceable standards. Across all analytes, average accuracy exhibited 4.10% error with an average precision of 4.28% RSD.

Table 3.3: Figures of analytical merit comparing concentration values obtained by S-SBSE-IDMS (n=5) in blank-subtracted serum with calculated concentrations. Unless noted, all concentrations are in units of μg/g

Analyte	†Log K_{ow}	†,‡Log VP	Calculated Value	§Experimental Value	|%Error|	%RSD	LOQ (ng/g)
Benzene	2.3	7.12	9.14±0.46	9.77±1.1	6.89	9.4	163
Toluene	2.69	6.47	6.34±0.31	5.93±0.39	6.47	5.3	29.7
o-Xylene	3.12	6.03	11.0±0.55	11.1±0.77	0.908	5.7	6.41
PCB-28	5.62	0.731	1.33±0.067	1.27±0.070	4.51	4.5	2.46
PCB-52	5.84	0.731	1.33±0.67	1.29±0.036	3.01	2.3	0.111
PCB-101	7.07	0.731	1.33±0.67	1.28±0.056	3.76	3.6	0.159
PCB-138	6.83	0.731	1.33±0.67	1.29±0.065	3.01	4.1	0.309
PCB-153	6.68	0.731	1.33±0.67	1.27±0.031	4.51	2.0	1.26
PBDE-47	6.81	-0.602	0.531±0.027	0.503±0.018	5.27	3.0	1.49
PBDE-99	6.5	-0.602	3.27±0.16	3.28±0.057	0.306	1.4	1.71
Chlorpyrifos	5.11	0.426	1.70±0.085	1.64±0.11	3.53	5.7	0.0648
Metolachlor	3.13	0.239	1.86±0.093	1.80±0.044	3.23	2.0	0.193
Acetochlor	3.12	0.656	2.10±0.11	2.25±0.23	7.14	8.5	30.1
Pendimethalin	5.18	0.669	0.641±0.032	0.624±0.0062	2.65	0.81	0.0621
DEHP	7.5	-1.08	0.610±0.030	0.562±0.075	7.87	6.1	1230

†Chemical values taken from material safety data sheets

‡Vapor pressure

§ Experimental values determined with n=5 replicates showing 95% CI

This research specifically focused on the quantification of a selected group of environmental toxins. However, the mechanism of SBSE is governed by the octanol-water partition ($K_{o/w}$) of an analyte, extraction phase volume, and sample volume.[2] It is, therefore, reasonable to propose that, using identical sample volumes and stir bars, this method could be expected to produce similarly high-quality data when expanded to analytes of $K_{o/w}$ values within the range of those included in this work (log $K_{o/w}$ 2.3 - 7.5). The validated method developed in this work was specifically optimized for the selected list of analytes; however, the mechanisms of extraction, separation, and quantification could allow a universal application to POPs of similar chemical characteristics.

3.4.6 Inter-Laboratory Analytical Method Transfer

Transfer of method quality to an independent laboratory, as demonstrated in figure 3.8, produced results that were statistically comparable to the results obtained at the primary laboratory at the 95% CI. A chemically diverse suite of analytes was chosen to test robustness of the method transfer. Total within-laboratory reproducibility for the primary laboratory across all selected analytes was 2.52% RSD; the independent laboratory achieved total within-laboratory reproducibility of 1.32% RSD.

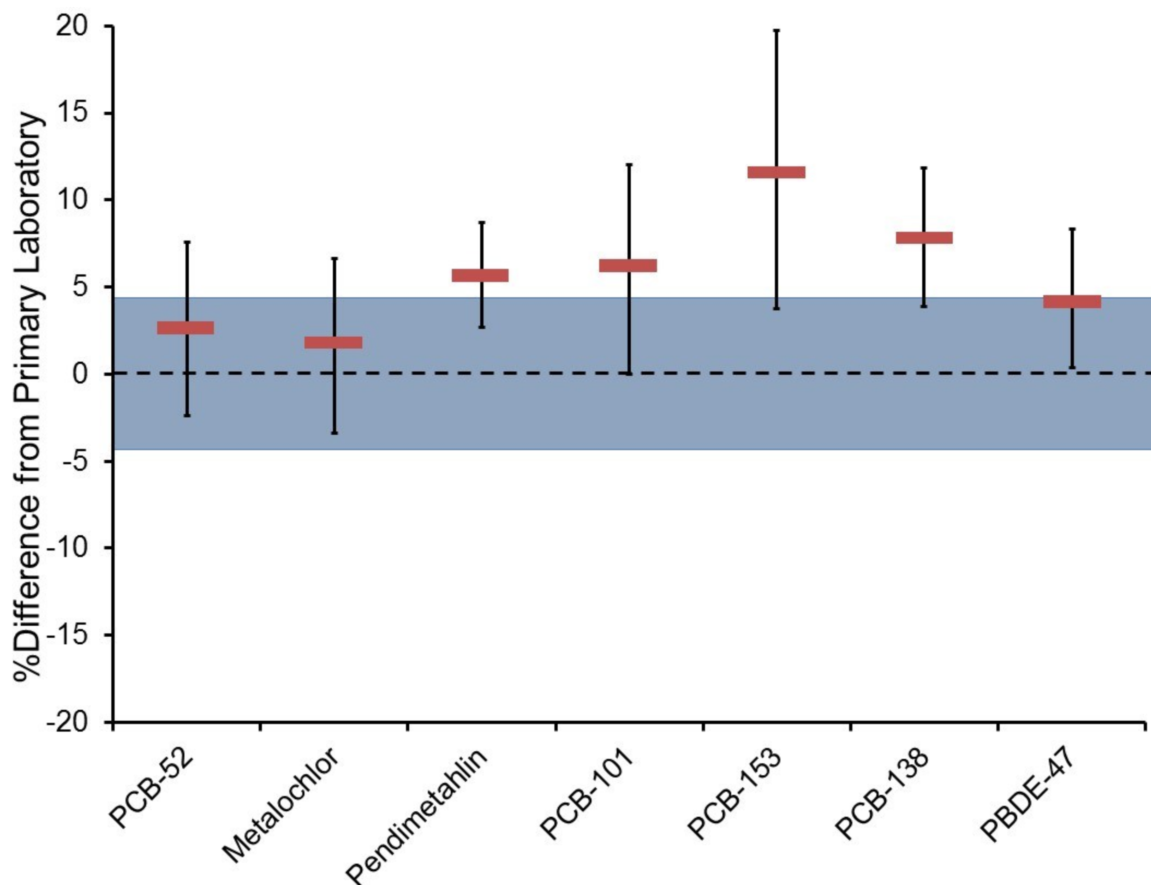


Figure 3.8: Percent difference in concentrations obtained for select compounds by a secondary analyst in an independent laboratory compared with the validation data obtained in the primary laboratory, showing 95% confidence of primary lab (shaded area) and secondary lab (bars)

In commercial, clinical, and government laboratories, inherent biological variability is often compounded by poor analytical reproducibility introduced by necessary, but complex, sample preparation steps.[13] In these labs, emphasis has been placed on development of simple analytical procedures that use small volumes of blood serum to obtain high accuracy, actionable results.[14] Such analyses require methods that are statistically accurate, highly reproducible, and efficient. Highly complex sample preparation steps may be reproduced by expert analysts. But, by compensating for much of the potential variance introduced in sample preparation, this method has demonstrated the potential to be transferred between laboratories and analysts with approximately two hours of operational training.

3.4.7 Assessment of Clinical Potential and Generalized Findings

The high accuracy and transfer capability of this optimized method are directly applicable to clinical chemistry, among other fields. As a proof of application, this section will detail analytical improvements in two recent IRB-approved collaborative studies with The Children's Institute of Pittsburgh (TCI) that investigated exposure to environmental toxins in children. In the first, existing methods using SPME were optimized for use with IDMS quantification. Multiple peer-reviewed sources claimed to improve sample-to-sample SPME reproducibility in blood serum.[15, 16] However, these methods were unable to be replicated in this laboratory for serum-immersive SPME analysis. The analytical incompatibility of SPME with immersive extraction from complex matrices such as serum and plasma necessitated the development of new methods for the analysis of POPs in small volumes of serum. The second study used SBSE and IDMS together to quantify a suite of organic toxins. Comparing the average quantifiable concentration of toluene, *o*-xylene, PCB-138, and PCB-153, (toxins included in both studies) figure 3.9 demonstrates the improvement in LOQ afforded by single bar SBSE-IDMS (mean LOQ=9.14 ng/g) over SPME-IDMS (mean LOQ=57.5 ng/g), which allowed quantification of biologically relevant concentrations of POPs. Analyzing by SPME-IDMSMS only quantified the greatest outliers.

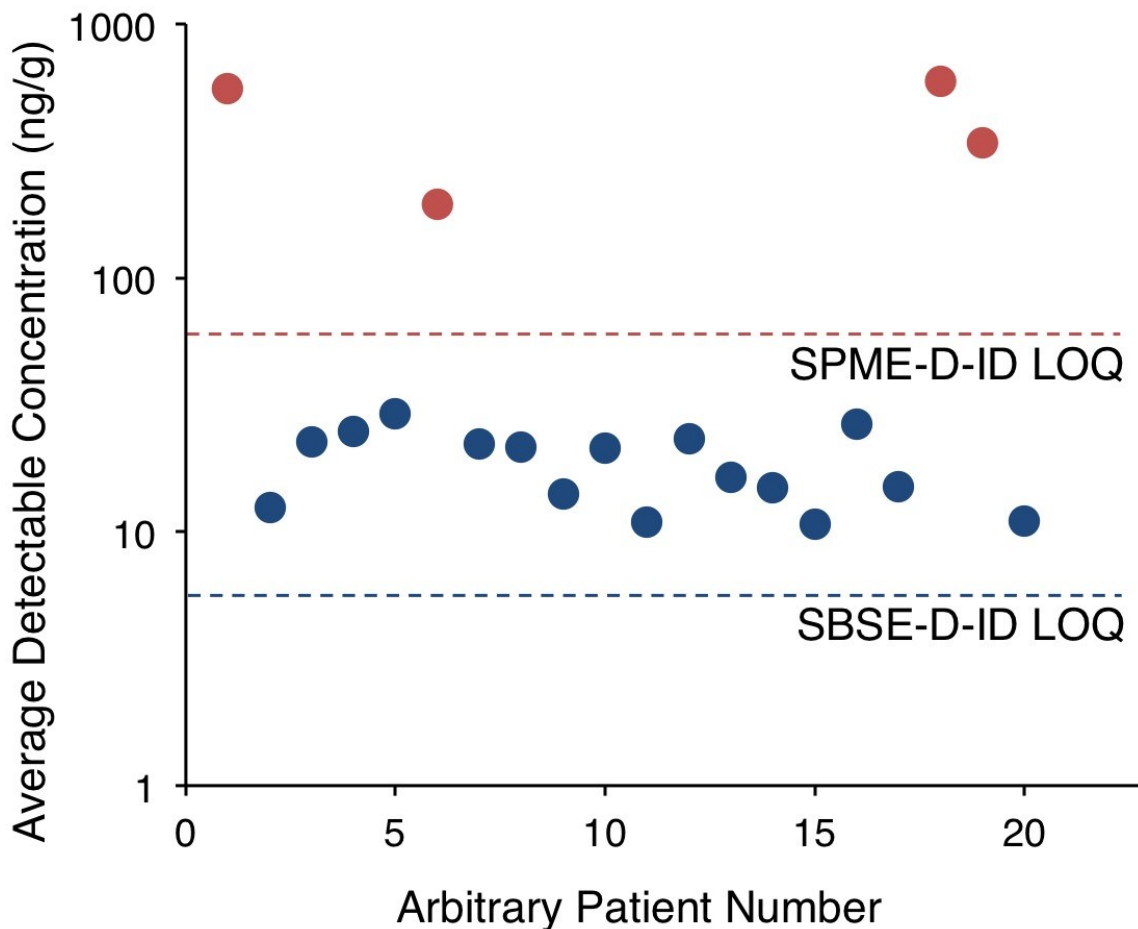


Figure 3.9: Mean toxin concentrations obtained using SBSE-IDMS, comparing mean LOQ of SBSE-IDMS and SPME-IDMS for POPs quantified in both studies.

To obtain actionable results and discern variable changes from clinical analyses, high quality data must be obtained with narrow confidence intervals. National and regional laboratories are often equipped to produce similarly high quality data for large populations. However, in this age of increasingly personalized medicine, local academic, clinical, commercial, and government laboratories must be capable of generating the same highly reliable and reproducible data. Observed in figure 3.10, using metolachlor as an example, this method allowed for the identification of statistically outlying individuals when population and sub-populations showed no statistical deviation from national average.

Figure 3.10: Mean difference from national average of metolachlor in regional collaborative study, 95% CI shown (n = 30x5, 27x5, 15x5, 5, respectively). Grey bar represents 95% confidence of national average.

The high throughput afforded by the increased efficiency and reduced sample preparation of this method enabled the generation of population and sub-population data for inter- and intra-comparison purposes. The reliability and sample-to-sample reproducibility of this method enabled high quality individualized analysis to be performed as well. The ability to reliably transfer among and between laboratories could allow local laboratories, with traditionally fewer resources than national laboratories, to generate the same high quality, reliable, and reproducible data.[17]

3.5 Application of Developed Method to Environmental Samples: EPA 625 Update

This section will describe experiments to improve upon existing EPA method 625, a standard method for the analysis of wastewater, by removing the necessity for large volumes of solvents required by outdated liquid-liquid extraction protocols of Method 625. Current methods for wastewater and drinking water are described including the research objectives for removal of liquid-liquid extraction and harmonization between differing methods and techniques.

3.5.1 Discussion of Wastewater Sample Preparation for GC-MS Analysis

The existing method for the quantification of organic pollutants in municipal and industrial wastewater was developed in the 1980s and requires the use of liquid-liquid or Soxhlet extractions.[18] Regardless of the scientific progress of the previous decades to reduce solvent consumption and waste generation, laboratories certified by the EPA for wastewater analysis are bound by the regulations of EPA Method 625, which requires 0.5L sample volumes and up to 250 mL of a liquid-liquid extracting solution containing hexane, methylene chloride, and acetonitrile.

The EPA has also produced methods for the quantification of organic pollutants in drinking water: EPA Method 525 (and the updated EPA Method 526).[19, 20] Method 525 removes the stipulation of liquid-liquid extraction and permits the use of solid-phase extraction columns or cartridges. While this has freed laboratories to remove the waste-generating liquid-liquid extractions, the title of the method still specifies liquid-liquid as the extraction technique. In the 1990s, the EPA developed and produced EPA Method 526, a method that specifically names SPE as the extraction technique of choice and removes "liquid-liquid extraction" from the Method title. EPA Method 525 contains a larger analyte-list by a significant margin and, as such,

is the Method utilized by a large number of laboratories. As the two methods exist for the quantification of chemically similar analytes, many commercial laboratories are only certified for Method 525. This means that, while Method 526 exists as a “greener” alternative to the waste-generating Method 525, additional and continued work is being done to include solid-phase extraction techniques into existing EPA Methods.

Much has been written already on the differences and similarities between Method 625 and 525, but several important differences must be noted.[21] The compound lists between the two methods are significantly different, however this is primarily a result of the times in which the two methods were developed, and not based in scientific need or industry regulation. Due to existing regulations, Method 625 analyte-list was never expanded. Instead, a third method was developed for a dramatically expanded list of organic pollutants in water, EPA Method 8270 for the analysis of groundwater and hazardous waste water.[22] As Method 8270 was based on Method 625 sample preparation protocol, albeit with a significantly expanded analyte-list, the stipulation for liquid-liquid extraction remained. While each of these three methods is overseen by a unique department within the EPA, 625 and 525 are both regulated by Section 304(h) of the CWA, further enabling their harmonization.[23] While similar in breadth and object, Method 8270 is a Resource Conservation and Recovery Act (RCRA) method of analysis, under SW-846 guidelines, and Methods 625 and 525 are governed by the office of water and Clean Water Act.. SW-846 is a compendium consisting of hundreds of methods and guidelines for analytical procedures governing the analysis of wastes.. The division between SW-846 methods and CWA methods has created a scenario in which standardized methods, with often similar aims, objectives, and analyte lists, can contradict one another in significant ways. Harmonization of standardized government methods is the ultimate objective of updating Method 625.

In the era in which these three Method were originally developed, fewer options existed for SPE of analytes of extremely high or low hydrophobicity, eliminating the incentive to remove liquid-liquid extraction- a method capable of extracting an extremely wide range of hydrophobicity analytes. More modern SPE technologies exist that are more capable of replicating the breadth, accuracy, precision, and sensitivity of liquid-liquid extractions.[24]

The purpose of this extended application of the developed S-SBSE-IDMS method described above was to demonstrate the feasibility of applying SPE techniques present in Method 525 to samples covered by Method 625, the first step in eventual method harmonization. Ultimately an updated 625 method will be produced with these SPE alternatives and lists of appropriate SPE materials, appropriate analytes and method parameters that enable this transition to SPE and a move away from older and less green liquid-liquid technology. The demonstration of SPE was accomplished via two experimental accomplishments. The first aim of these research demonstrations highlighted the robustness of the developed S-SBSE-IDMS method by the production of validated analytical data in wastewater (a separate yet complex solution from blood serum). The second demonstration and aim of this work was to provide recommendations to the EPA, through an involvement with a collaborative working-group consisting of 23 laboratories from government, industry, and academia, on the update of EPA Methods 625. To accomplish these aims, the previously developed S-SBSE-IDMS method was validated for the differing analytes and potential matrix effects using EPA recommended validation parameters. Second, the validated method was assessed for feasibility of inter-laboratory method transfer. Finally, the method was compared against the methods developed by collaborative working group as a whole and with the other working group laboratories using SBSE to gauge the effectiveness of S-SBSE-IDMS.

3.5.2 Methods and Instrumentation

For method development, the total analyte list from EPA 625 was reduced to a list of 21 compounds, representing the wide range of chemo-physical differences present in the full analyte list. For method transfer experiments, two sets of randomly selected analytes were prepared into solution of synthetic wastewater and shipped, with all required GC/MS and quantitative software methods and standard operating procedures, to two independent laboratories with separate analysts for comparison with results achieved by the author in the primary laboratory at Duquesne University.

Instrumental parameters were set to the recommendations of EPA Method 625. Extraction and quantification procedures were described previously for S-SBSE-IDMS of blood serum samples. The instrumental steps were partially automated using the GERSTEL MPS II, Agilent 7890A GC and an Agilent 5975C mass spectrometer. Agilent Masshunter software was used to determine the peak area of each analyte and its isotopically labeled counterpart at the quantitative m/z ions listed in table 3.4. Quantitative *m/z* values for the natural analytes were acquired from EPA Method 625 and isotopic *m/z* values were acquired experimentally. Using acenaphthylene and $^{13}C_8$-acenaphthylene at examples, figure 3.10 demonstrates the mass spectra corresponding to a naturally occurring molecule (a), isotopically enriched analog (b), and a mixture of both natural and isotopically enriched.

Compound identification was performed by analysis of mass spectra for the quantitative and confirmatory *m/z* fragments of the analytes of interest. Identification of quantitative and confirmatory peaks for isotopic analogs involved altering the quantitative *m/z* spectral peak of the naturally occurring compound by the addition of mass units equal to the number of isotopic enrichments located on the quantitative molecular fragment. For an example, figure 3.11 shows

the identification of acenaphthylene by the presence of the quantitative *m/z* peak at 152 and the confirmatory peak at 153 *m/z*. The mass spectrum for $^{13}C_8$-acenaphthylene (middle) was identified by the quantitative peak at 160 *m/z* and the confirmatory peak at 161 *m/z.* A mixture of the two analogs (bottom) displays both quantitative peaks and confirmatory peaks.

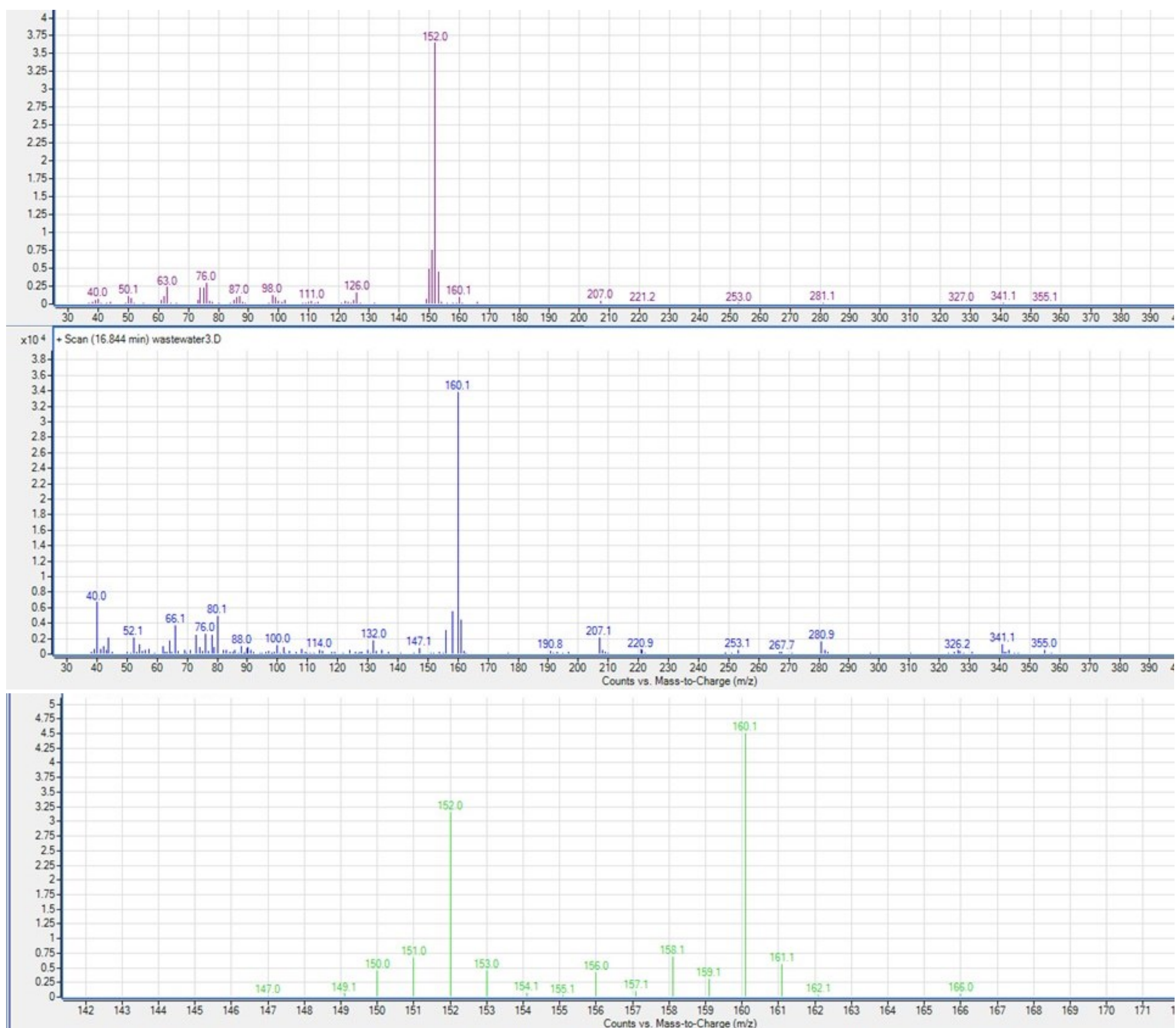


Figure 3.11 Mass spectrometric analysis showing the quantitative and confirmatory *m/*z peaks of (top) naturally occurring acenaphthylene, (middle) isotopically enriched $^{13}C_8$-acenaphthylene, and (bottom) a mixture of both analogous forms of acenaphthylene

Table 3.4: Quantification ions for natural and isotopic molecules with respective chromatographic retention times (R.T.)

Analyte	Quantitative Ion (m/z)		Approximate R.T. (minutes)
	Isotope	*Natural*	
B-BHC	187	181	21.6
1,2,4-Trichlorobenzene	183	180	12.5
4,4-DDD	243	235	27.7
4,4-DDT	247	235	28.6
Acenaphthene	164	154	17.6
Aldrin	270	263	24.4
Anthracene	184	178	22/22.1
Benzo[a]pyrene	264	252	33.4
Benzo[k]fluoranthene	264	253	32.7/32.6
Benzyl buty lphthalate	264	253	32.7/32.6
Bis(2-ethylhexyl)phthalate	153	149	30.45
Di(benzo[a,h])anthracene	292	278	35.7
Diethyl phthalate	125	121	19.29
Dimethyl phthalate	167	163	17.17
Fluroranthene	172	166	19.2
Hexachlorobenzene	290	284	21
Hexachlorobutadiene	229	225	13.1
2-Chloronaphthalene	170	162	15.9
Pyrene	208	202	42.7
Naphthalene	134	128	12.67
Phenanthrene	184	178	22/22.1

3.5.3 Wastewater Validation Results

Samples of synthetic wastewater spiked at unknown concentrations of all listed analytes were sent to all working-group participating laboratories for validation of various SPE techniques. Figure 3.12 shows the accuracy and precision achieved by the S-SBSE-IDMS compared with the "actual" values obtained by the EPA certified laboratories using EPS Method 625 by plotting percent difference of the experimental values obtained by S-SBSE-IDMS from the EPA-certified laboratory. EPA-certified results were used for comparison and validation. Using these results, however, provided an additional level of validation for SBSE-IDMS. The EPA-certified laboratories (upon which the "actual" values were based) achieved a mean precision (95% confidence) of ±54.1%, while the S-SBSE-IDMS method in this laboratory

produced a mean precision (95% confidence) of ±17.6%. A significant finding in this work was that IDMS, when compared with the EPA-certified laboratories using EPA Method 625, generated only positive bias in quantitative error. The source of this bias, whether due to a bias in the experimental method or due to error introduced by the EPA laboratories, will be investigated in future work.

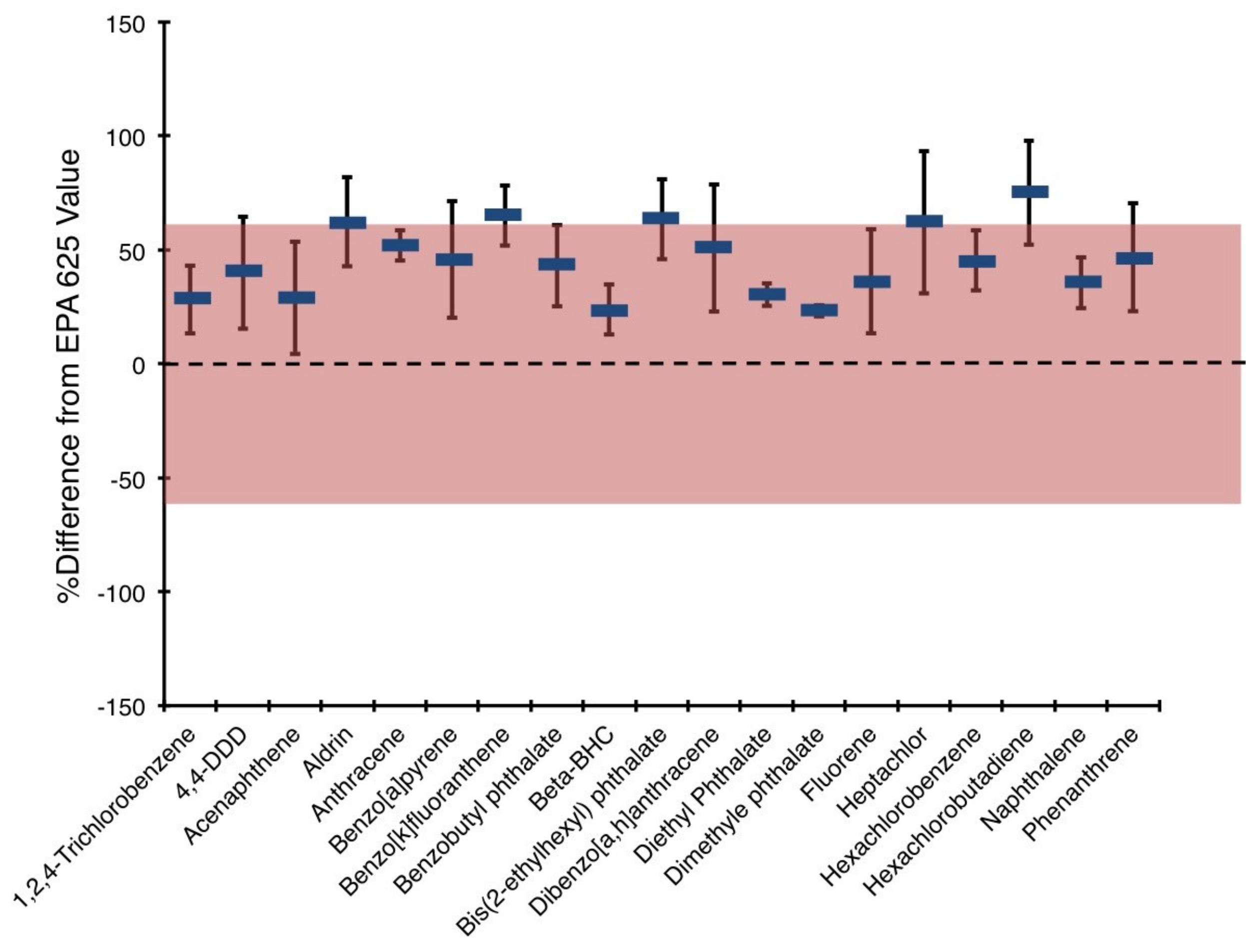


Figure 3.12: Mean concentration values obtained using the developed S-SBSE-IDMS method, showing percent difference from "actual" value obtained by EPA certified laboratories using EPA Method 625, showing 95% confidence limits of the mean from this lab (bars) and the mean precision of the EPA-certified laboratories (shaded area: 54.1%)

The accuracy and precision values obtained using S-SBSE-IDMS fall within the stipulated regulations enforced by the EPA. For each analyte listed in EPA Methods 625, 525, and 8270, analyte-specific QC acceptance criteria are provided within the method. Mean accuracy required for Method 625 is approximately ±60% error, with mean required precision approximately ±30% RSD (and up to 98% RSD for many analytes). These acceptance criteria are very broad and can likely be improved with the addition of up-to-date analytical techniques and quantitative procedures. Validation figures can be found in table 3.5. The validated method presented here was capable of significantly improving the precision of quantification at 95% confidence, from ±54.1% to ±17.6%, a significant difference at p=0.0223.

Table 3.5: Validation figures for the quantification of selected EPA 625 analytes in synthetic wastewater, showing %difference from EPA-certified values

Analyte	%Difference	%RSD	LOQ (ng/mL)	LOD (ng/mL)
1,2,4-Trichlorobenzene	28.2	10	2.98	1.79
4,4-DDD	40.1	17	22.16	13.3
Acenaphthene	29.0	17	6.08	3.65
Aldrin	62.3	13	3.23	1.94
Anthracene	51.9	4.5	2.95	1.77
Benzo[a]pyrene	45.7	18	20.26	12.16
Benzo[k]fluoranthene	65.1	12	41.23	24.74
Benzobutyl phthalate	43.1	12.	17.2	10.32
Beta-BHC	23.7	7.5	15.53	9.32
Bis(2-ethylhexyl) phthalate	63.5	12	18.01	10.81
Dibenzo[a,h]anthracene	50.8	19	35.11	21.07
Diethyl Phthalate	30.4	3.4	151.8	91.08
Dimethyle phthalate	23.3	1.7	2.45	1.47
Fluoranthene	36.2	16	2.61	1.56
Heptachlor	62.2	21	2.72	1.63
Hexachlorobenzene	45.5	9.0	1.14	0.68
Hexachlorobutadiene	75.2	18	3.13	1.88
Naphthalene	35.5	7.6	1.64	0.98
Phenanthrene	46.9	16	2.22	1.33

%Difference: Percent error from the concentrations generated by EPA-certified labs
LOQ: Limit of quantification as defined by a 5:1 signal to noise ratio.
LOD: Limit of detection as defined by a 3:1 signal to noise ratio.

3.5.4 Inter-laboratory Analytical Method Transfer

Two sets of synthetic wastewater were prepared in the primary laboratory and spiked with randomly selected amounts of ~10 analytes chosen from the EPA Method 625 analyte list. Figure 3.8 shows the results of the first laboratory method transfer experiment via flash storage device containing GC/MS and quantitative software and standard operating procedures. In figure 3.13, percent difference is shown between concentration values obtained by the independent laboratory compared with the primary laboratory. The transfer was highly effective, producing 6.85% mean difference from the primary laboratory results, and 7.95% mean intra-laboratory %RSD. Quantifying this reduced analyte list, the primary laboratory produced a mean intra-laboratory precision of 2.11% RSD.

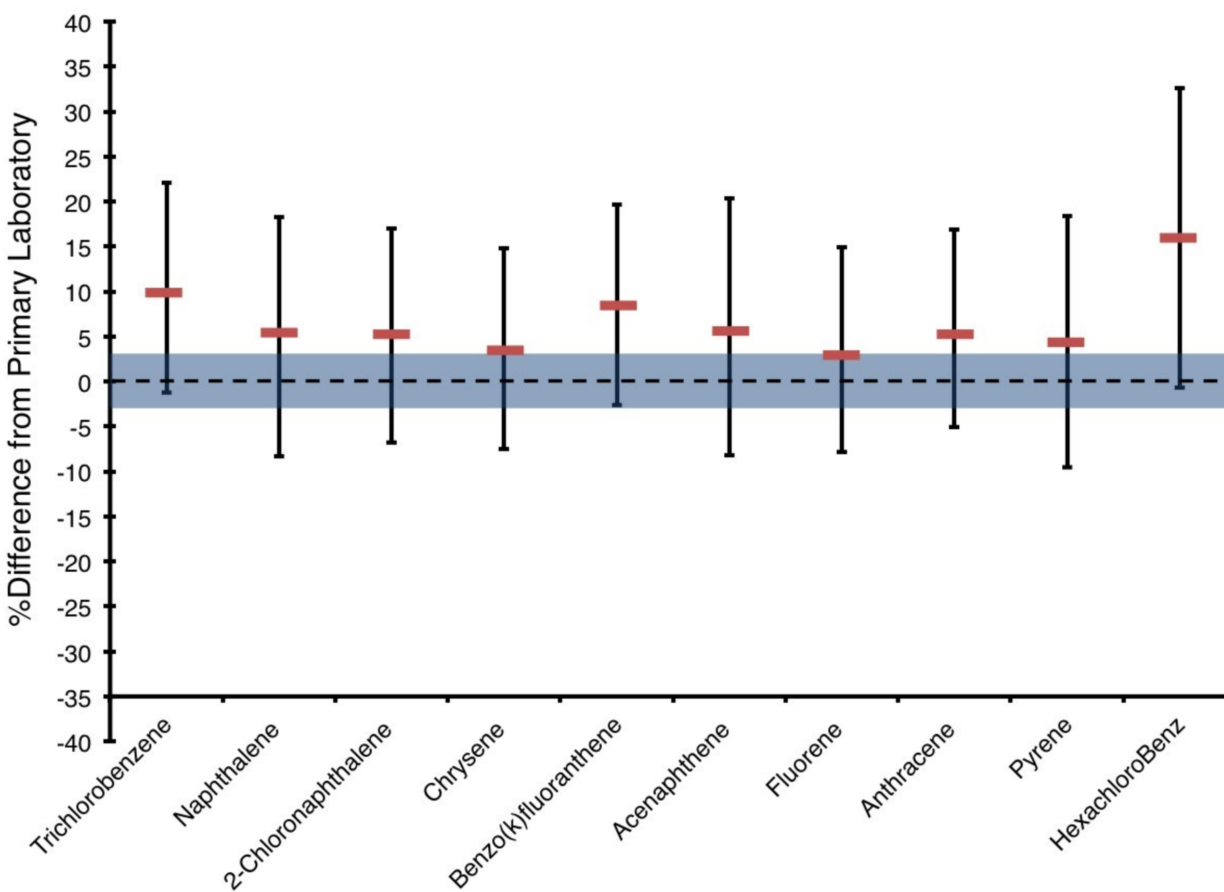


Figure 3.13: Demonstration of effective laboratory method transfer into a second laboratory, showing percent difference from concentrations obtained in the primary laboratory and 95% confidence levels. Mean 95% confidence achieved in the primary laboratory is shaded in blue.

A second method transfer experiment was performed by again transferring both GC/MS methodology/quantitative software and standard operating procedure to an independent laboratory with a unique analyst (different from transfer experiment 1). Results of this method transfer experiment can be found in figure 3.14. Mean percent difference from the primary laboratory concentration values was 6.28%, with a mean intra-laboratory %RSD of 5.10%. For this set of analytes, the primary laboratory produced a mean intra-laboratory precision of 5.02%. Both method transfer experiments produced quantitative values that were statistically indistinguishable from the primary laboratory, effectively transferring the expertise of the

primary laboratory digitally. The differences observed in the secondary and tertiary laboratories from the primary laboratory were equally divided between positive and negative differences, indicating no distinct positive or negative bias. Both sets of method transfer experiments resulted in significant positive error, with the receiving laboratory reporting quantitative data with positive error in all cases. The source of this bias, being non-random in nature, will be investigated in future research. One potential source of this bias is the loss of natural analyte by glass-wall adhesion during shipment to the receiving laboratory, resulting in uniform positive bias in all samples analyzed by receiving laboratory.

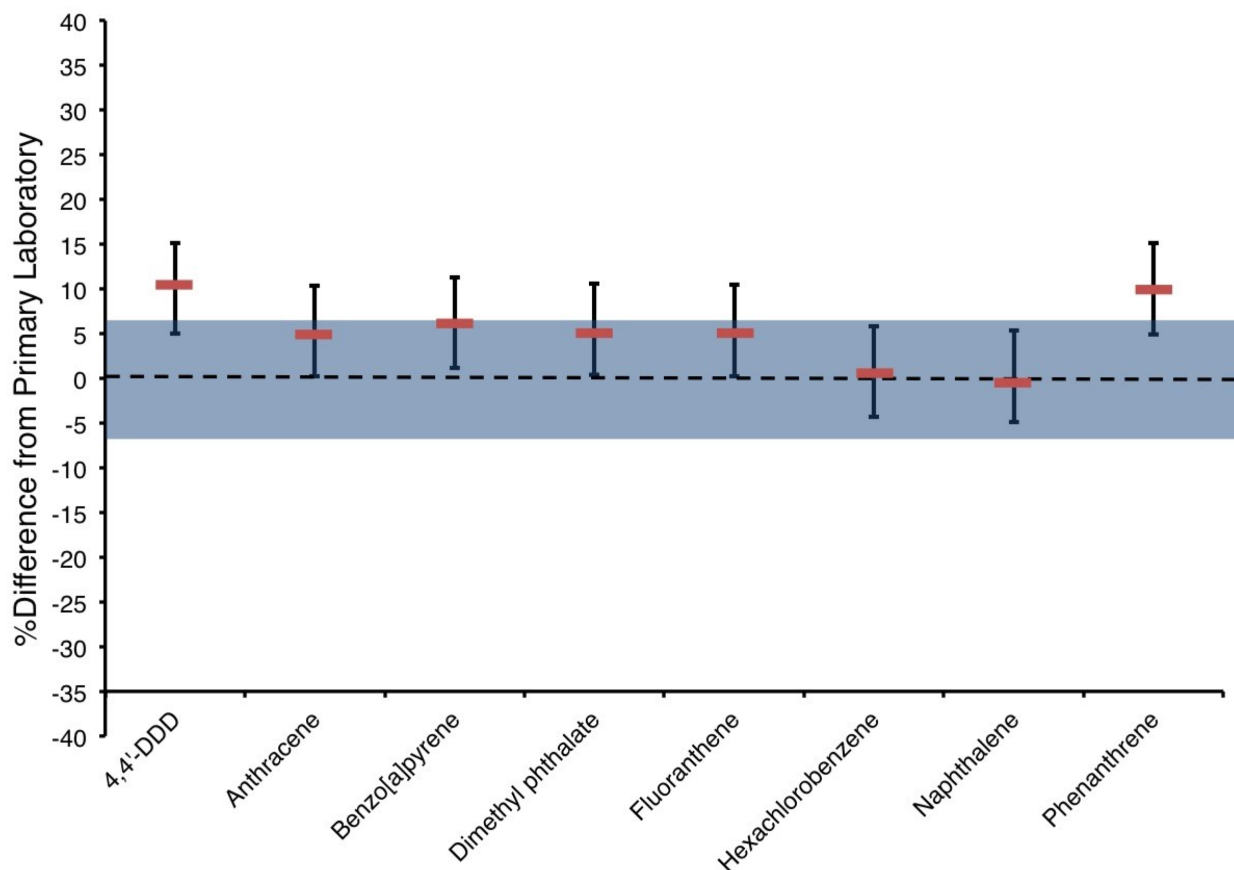


Figure 3.14: Figure 3.8: Demonstration of effective laboratory method transfer into a third laboratory, showing percent difference from concentrations obtained in the primary laboratory

and 95% confidence levels. Mean 95% confidence achieved in the primary laboratory is shaded in blue.

3.5.5 Analysis of Unknown Wastewater Samples

The developed method, validated against EPA-certified concentrations, was applied to the analysis of wastewater samples spiked with unknown concentrations of EPA Method 625 analytes in a blind study performed by the collaborative working-group. The accuracy achieved in this work surpassed the accuracy achieved by laboratories analyzing via SBSE-calibration curve, but this improvement in accuracy was not statistically significant ($p=0.643$). This laboratory achieved higher accuracy quantification than the rest of the pooled laboratories that used traditional SPE columns and cartridges ($n=21$) at a statistically significant level ($p=0.019$).

All laboratories using SBSE, including IDMS and calibration curve quantifications, achieved higher mean accuracy than the rest of the SPE laboratories, but outside of the cut-off for statistical significance ($p=0.068$). These results indicate that SBSE may provide an improvement in accuracy over traditional SPE for the analysis of synthetic wastewater by EPA Method 625, but only when SBSE was combined with IDMS were the improvements in accuracy over traditional SPE statistically significant. Table 3.6 shows a comparison of quantitative accuracy for analytes that were quantified by all laboratories. As many laboratories did not report values which fell below laboratory-specific LOQ values, only the analytes for which complete data-sets were available were chosen for this comparison as to not bias the results.

Table 3.6: Comparison of method accuracy against a laboratory analyzing by SBSE, and against the rest of the study

Analyte	%Mean Difference from EPA-Certified Value		
	Duquesne	*SBSE-Lab*	*All Labs*
Acenaphthene	15.8	1.28	29.4
Anthracene	4.27	42.2	41.7
Hexachlorobenzene	4.47	8.59	35.9
Benzo(k)fluoranthene	18.7	26.4	29.1
Di-n-butylphthalate	31.5	21.8	23.8
1,2,4-Trichlorobenzene	37.7	31.3	39.8
Pyrene	4.35	10.2	26.5
Mean	**16.7**	**20.2**	**32.3**

The quantification values achieved by this laboratory were produced in three different water matrices: synthetic wastewater (WW), synthetic wastewater with 20% acetonitrile matrix modification (WW+ACN), and drinking water (DI). The three matrices produced statistically similar concentration values for all measured analytes, excluding the analyte isophorone. Figure 3.15 shows a comparison of the quantitative values achieved by this laboratory in three separate matrices, showing statistically significant agreement. Isophorone was not an EPA 625 compound spiked into the unknown mixture. It is primarily used as a solvent in printer ink, paints, adhesives, lacquers, coatings, and finishings and was discovered to be a significant contaminant in the Duquesne laboratory, but not in either of the method transfer receiving laboratories. Routes of contaminant introduction in the primary laboratory were limited due to the filtration system instrumental enclosure installed around the GC/MS and sample handling system. The likely introduction route was during the sample preparation step when the sample was exposed to ambient, unfiltered laboratory air. Isophorone was not present in the coatings applied to the flooring, the wall paint, and the lacquer used on the filter system enclosure. Future sample preparation work must be performed in the ISO Class 5 cleanroom to prevent further sample contamination.

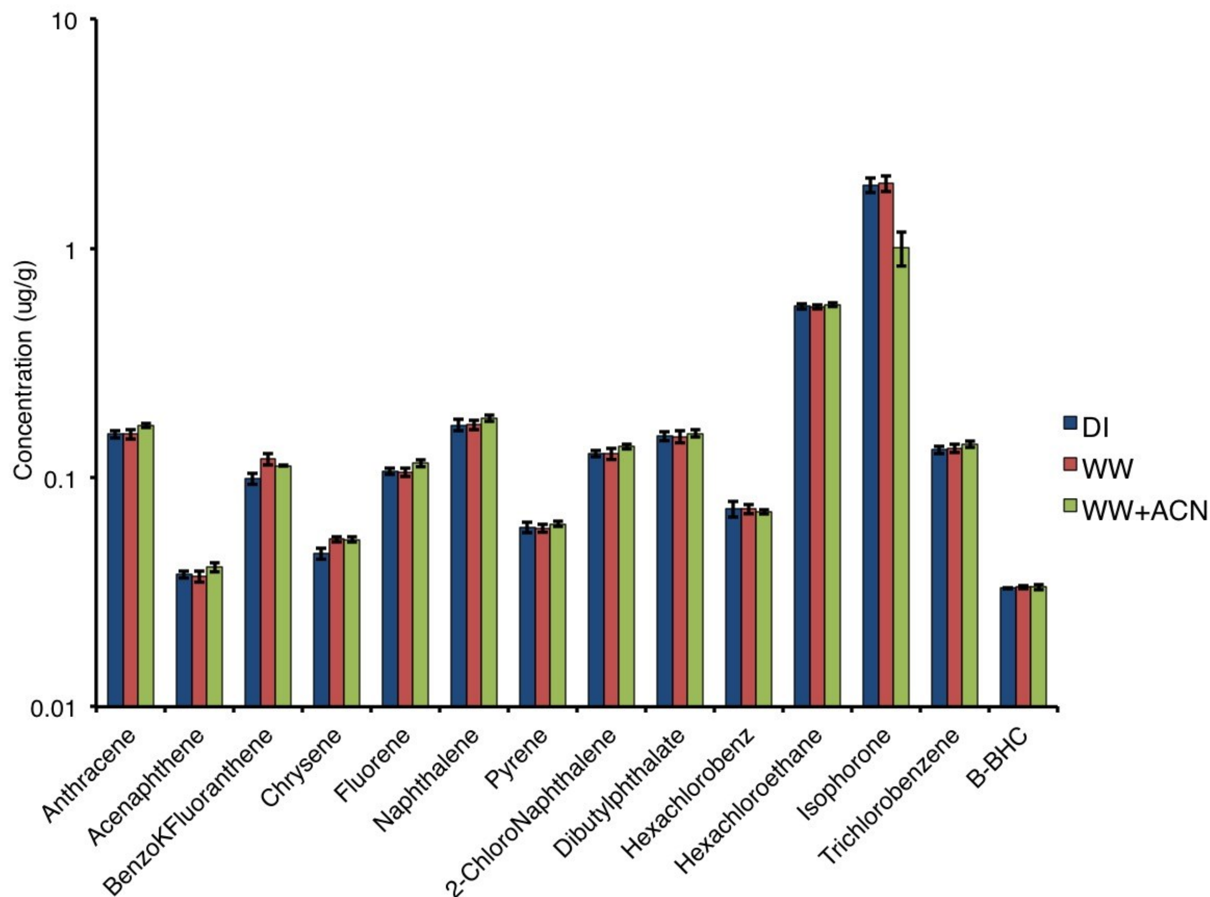


Figure 3.15: Quantitative values achieved for the studied EPA 625 analytes in three separate matrices, showing 95% confidence. The contaminant isophorone was quantifiable in all three matrices in the primary laboratory at Duquesne, but not at either receiving laboratory

The three solutions were extracted using identical methods for determining matrix effects. The similarity between drinking water recovery and wastewater recovery suggests that the method was able to correct for any wastewater matrix effects that may have been present. The comparison between WW and WW+ACN samples was important to determine if the WW samples were being biased by the presence of a immiscible solvent from the isotope-spiking procedure. The WW+ACN samples eliminated the single droplet of immiscible solvent but produced a cloudy solution, suggesting that the immiscible solvent did not dissolve, but simply created an emulsion of suspended micro-droplets in the solution. While further work is necessary

to determine the exact effects of the immiscible solvent and of the acetonitrile matrix modification, both WW and WW+ACN produced statistically indistinguishable quantitative results. This effect is discussed in detail in Section 3.4.6.

3.5.6 Limitations and Future Outlook

The primary limitation to this work that may have contributed to the relatively poor sensitivity of the highly hydrophobic compounds was the spiking of isotopic solution for IDMS into the aqueous samples. Care was taken in this work to maximize the concentration of isotopic stock solutions to minimize spike volume; however, due to the highly hydrophobic nature of many of the EPA Method 625 compounds, stock preparation required the use of hexanes and toluene for dilutions. Research was performed on stock preparation using the aqueous-miscible solvents acetone, methanol, and acetonitrile. However, the low $K_{o/w}$ of these solvents prevented the solvation of high concentrations of many EPA Method 625 analytes. Additionally, these solvents would not provide stability of time during storage, as the interaction between the glass container and the analytes would be stronger than that of the solvent and analyte.

Upon spiking microliter volumes of stock isotopes into the wastewater or drinking water samples, a small volume of immiscible solvent tended to form at the top of the solution. Theoretically, this immiscible solvent possessed similar properties to single-droplet extraction methods. Many of the analytes of interest in this research likely equilibrated between the aqueous solutions, the immiscible droplet, and the stir-bar, reducing the overall amount of compound present in the stir bar. As discussed in Section 3.4.5, the WW and WW+ACN samples produced statistically identical results, indicating that the solvent droplet did not influence the recovery, that IDMS was able to correct for the reduced recovery, or that matrix modification by

acetonitrile had no effect on improving recovery. This is not the only reasonable explanation for the relatively poor sensitivity of the highly hydrophobic compounds however. Incomplete thermal desorption from the stir-bar, particularly of the high $K_{o/w}$, low volatility compounds, could also reasonably explain the observed issues with sensitivity. While the LOD and LOQ for all studied analytes fell well below the required values specified in EPA Method 625, future studies on SBSE-IDMS with highly hydrophobic compounds should consider further investigation of the effects of spiking immiscible solvents into aqueous solution.

3.6 Conclusions

This S-SBSE-IDMS method was the first to combine the enhanced analyte recovery and sensitivity afforded by SBSE with the improvements in accuracy and precision attained by IDMS quantification. This method was novel in that it was the first to simultaneously extract both volatile and nonvolatile environmental pollutants simultaneously from microliter volumes of serum. This was a major improvement over existing methods, allowing researchers to reduce sample consumptions and improve the efficiency of consumable usage. The developed method was optimized and validated for biological research on blood serum to determine the organic pollutant body-burden of an individual using a high-quality analytical method. Comparing this validated method with existing, industry-accepted method like SPME and SPME-calibration curve demonstrated the significant improvement afforded by S-SBSE-IDMS in both its potential as a research tool and in a practical clinical application.

The validated S-SBSE-IDMS method was the applied to a separate, but similarly complex, sample: industrial wastewater. While the sample matrix used for this research was synthetic wastewater, the matrix possessed many of the confounding characteristics of real world

wastewater. The S-SBSE-IDMS method produced EPA-validated quantification results of higher quality than all other laboratories using similar SPE technologies. The high-quality data produced by this method in analysis of unknown wastewater samples will be included in recommendations to the EPA, in collaboration with a 23-laboratory working-group, on methods and techniques to effectively update the regulations and standards for industrial and municipal wastewater analysis, to align these regulations more closely with current drinking water analysis standards.

This method was validated for the analysis of environmental pollutants in two unique, but equally complex, sample matrices. In both sets of experiments, the analytical quality of the S-SBSE-IDMS method surpassed existing methodologies and technologies. Likewise, specific emphasis was placed on eliminating complex sample preparation steps to produce a final method capable of being transferred between laboratories and analysts while maintaining analytical quality. This research has produced a highly accuracy, highly precise analytical method capable of maintaining analytical quality when transferred digitally between and among laboratories and analysts. These method characteristics are invaluable to future work in both clinical and environmental analyses.

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Chapter 4: Relationship between organic pollutants and autism severity in children with autism and neurotypical peers

4.1 Introduction

Emergent data have implicated environmental exposure, evidenced by serum-concentrations of specific environmental pollutants, as a correlative factor in the etiology of numerous non-communicable diseases, such as autism spectrum disorder (ASD), heart disease, diabetes, and lupus. [1-4] Exposure to heavy metals and organic pollutants, collectively termed xenobiotics, has been linked with neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, suggesting the central nervous system is specifically susceptible. [5, 6]

Much environmental and exposomic research is epidemiological in nature. Concern has been expressed about agreement between self-reported and clinically assessed values in epidemiological studies, particularly with rare health events. [7, 8] Many studies tend to rely upon proxy measurements, such as written surveys, interviews, or self-report, to establish exposure. Studies have found that the use of proxies can introduce bias into methodological outcomes when self-reported measurements are used in lieu of direct clinical assessments. [7, 9] While epidemiological and exposomic studies have suggested that neurological symptoms can be correlated with pre- and post-natal exposure to environmental pollutants, direct quantitation of environmental pollutants is necessary to eliminate the potential for proxy bias.

In biological fluids, analytical quality is often hindered by inherent biological variability as well as complex sample preparation and analytical procedures. Further, calibration curve and internal standard quantifications are inherently unable to correct for sample-to-sample variability and errors introduced into quantification by extraction, sample preparation, and instrumental

analysis. This quantitative variability can be improved using direct quantitation techniques like IDMS. [10, 11] High-throughput sampling and direct-quantitation methodologies were utilized in this work to achieve high accuracy and precision and to correct for error introduced by sample preparation, chromatography, and mass spectrometry. These technologies allow researchers to accurately quantify environmentally relevant concentrations of organic pollutants. [11, 12]

4.1.1 Hypothesized Autism Spectrum Disorder Pathophysiology

A hypothesis has been proposed that suggests a portion of the pathophysiology of ASD may relate to interactions between physiology and the environment in genetically predisposed or otherwise susceptible individuals.[13, 14] A fundamental expectation in this hypothesis is the observation of differential physiological response to similar environments by susceptible individuals compared with typical controls. A number of pathways have been proposed to explain this physiological/environmental interaction hypothesis, focusing primarily on impaired methylation and detoxification that can lead to glutathione-redox imbalance and central nervous system excitotoxicity. Studies have discovered each of these features in individuals with ASD. [13, 15-18]

Methionine (Met) is an essential amino acid that acts as a methyl group donor to facilitate cellular methylation to, in part, protect, stabilize, and epigenetically activate or deactivate DNA. Environmentally-sourced xenobiotics have been shown to inhibit the activity of methionine synthase (MS) [19], the enzyme responsible for converting homocysteine to Met. Exposure to some xenobiotics may, therefore, reduce cellular methylation capabilities in numerous systems that require Met. One important methylation pathway is dopamine-stimulated phospholipid methylation (PLM), which utilizes a D4 subtype dopamine receptor that is critical for

synchronization of brain activity during attention. [20, 21] This suggests that xenobiotic impairment of MS could limit dopamine-stimulated PLM, leading to impairments in neuronal synchronization and attention, key features found in children with ASD. [13, 22]

Detoxification is the process by which xenobiotics are removed from the body and is dependent on Met and MS for many necessary functions. The primary antioxidants, Met and glutathione (GSH), are sulfur-containing antioxidants that act as a primary pathway for removal of xenobiotics from tissues, regardless of chemical identity. [23] Therefore, overall stress on detoxification pathways may be more dependent on overall xenobiotic insult than on specific xenobiotics. [13] Impaired MS activity, therefore, may limit the detoxification capacity of the body by reducing sulfur-containing antioxidant pathways.

Met displays moderate antioxidant capacity and acts as a biochemical precursor to the primary bodily antioxidant, GSH. Balance between reduced glutathione (GSH) and oxidized glutathione (GSSG) has a broad impact on overall cellular health. Met protects GSH/GSSG redox balance by preventing GSH depletion, as GSH is synthesized in a reaction involving Met. An imbalance in GSH/GSSG and an increase in reactive species in the body is called oxidative stress. Oxidative stress can allow xenobiotics, specifically organochloride pesticides and polychlorinated biphenyls, to disrupt the neurotransmitters glutamate and gamma-aminobutyric acid (GABA) and lead to central nervous system excitotoxicity, which can exacerbate symptoms of ASDs. [15, 24] This suggests that impaired MS activity could create competition for available GSH in the GSH/GSSG redox-cycle, [25] resulting in increased oxidative stress and greater potential for central nervous system excitotoxicity.

Considering these atypical pathways discovered in individuals with ASD, the proposed physiological/environmental interaction hypothesis suggests that a feedback cycle, triggered by

environmental insult, may impair MS activity and affect multiple systems simultaneously by decreasing methylation capabilities, inducing or exacerbating detoxification dysfunction, limiting dopamine-stimulated PLM, and ultimately promoting excitotoxicity, leading to neurological symptoms associated with ASD.

While some studies have implicated specific xenobiotics as correlative factors in ASD etiology, this study sought to explore the combinative physiological effects of environmentally relevant concentrations of multiple organic pollutants. With this objective, direct chemical measurements of non-metabolized organic pollutants and behavioral rating scales/observations were conducted in children diagnosed with ASD (n=30) and controls (n=30) matched by age, sex, and socio-economic status in Western Pennsylvania to investigate the relationship between organic pollutants and neurological symptoms associated with ASD.

4.2 Materials and Methodsfor the Study of Children with Autism and Controls

The study was approved by the Institutional Review Board (IRB) of Duquesne University (Pittsburgh, PA, USA) (IRB #10-36) and was also conducted at The Children's Institute of Pittsburgh. All analysts involved with sample processing and chemical quantitation remained blind to the identification and ASD-diagnosis status of all individuals throughout the course of the study and data analysis.

4.2.1 Analyte Selection

Previous studies guided the selection of organic xenobiotics associated with etiology of ASD. [14, 26] The selected analytes represented a wide range of volatility, chemical function, and hydrophobicity. Three volatile organic compounds (VOC), benzene, toluene, and *o*-xylene; two

alkanes, pentane and hexane; five polychlorinated biphenyls (PCB), IUPAC congeners 28, 52, 101, 138, and 153; two polybrominated diphenylethers (PBDE), IUPAC congeners 47 and 99; two organochlorine pesticides, metolachlor and acetochlor; one dinitroanaline pesticide, pendimethalin; one organophosphate pesticide, chlorpyrifos; one phthalate, bis(2-ethylhexyl) phthalate (DEHP); and the chlorocarbon perchloroethylene were chosen for this research. Further information concerning analyte selection is provided.

VOC and alkane xenobiotics are byproducts of incomplete combustion and are typically markers of automobile exhaust. PCB production was banned in the U.S. in 1979 but the ubiquitous nature and environmental persistence of PCBs has resulted in measurable environmental background concentrations and acutely contaminated sites have been discovered in recent years. The two organochlorine pesticides, metolachlor and acetochlor, are the most applied pesticides in Western Pennsylvania by weight, used primarily on corn and soybeans. Chlorpyrifos and pendimethalin are also highly used pesticides and they represent modes of biological activity separate from the organochlorine pesticides. PBDEs are flame retardant compounds used in furniture cushioning and carpeting. DEHP is a plasticizer widely used in the production of PVC, glow sticks, and linoleum flooring.

4.2.2 Quantitative Methods and Instrumental Protocols

Serum samples were analyzed by stir-bar sorptive extraction. Chromatography and mass spectrometry were conducted using an Agilent 6890 Gas Chromatograph – 5975 Mass Spectrometer system (Agilent Technologies, Santa Clara, CA, USA) with a non-polar column (HP-5). Analytes were introduced into the GC-MS thermally using a thermal desorption unit (GERSTEL GmbH & Co., Mülheim, Germany). Method blanks were run between replicates and

instrument blanks between each new sample. Quantitation was accomplished using the standard method of isotope dilution-mass spectrometry (IDMS). [10] Complete reagent, instrumental, and quantitative materials and methods are provided.

4.2.3 Reagents

A standard containing six polychlorobiphenyl (PCB) congeners [2,4,4'-PCB (PCB-28), 2,2',5,5'-PCB (PCB-52), 2,2',4,5,5'-PCB (PCB-101), 2,2',3,4,4',5-PCB (PCB-138), 2,2',4,4',5,5'-PCB (PCB-153), and 2,2',3,4,4',5,5'-PCB (PCB-180) as well as standards for polybrominated diphenyl ether (PBDE) congeners [2,2',4,4'-BDE (PBDE-47) and 2,2',4,4',5-BDE (PBDE-99)] all at certified concentrations were purchased from Sigma-Aldrich (St. Louis, MO). Benzene, toluene, *o*-xylene, (≥99% purity) and bis(2-ethylhexyl) phthalate (DEHP) were purchased from Fluka (St. Louis, MO) at certified concentrations. Certified standards for chlorpyrifos (99.5% pure), pendimethalin (98.8% pure), acetochlor (98% pure), metolachlor (98.6% pure), toluene-d_8, *o*-xylene-d_{10} were obtained from SPEX CertiPrep Group (Metuchen, NJ). A certified standard for benzene-d_6 was obtained from Cerilliant (Reston, VA). Chlorpyrifos-d_{10} (99% labeled), tetrachlorethylene-$^{13}C_2$ (PCE) a standard mixture of PCB-$^{13}C_{12}$ (99% labeled, and standards for PBDE-47 and 99-$^{13}C_{12}$ (99% labeled) were obtained at certified concentrations from Cambridge Isotope Laboratories, Incorporated (Tewksbury, MA). Pendimethalin-d_5 (98% labeled), acetochlor-d_{11} (98% labeled), metolachlor-d_6 (98% labeled), bis(2-ethylhexyl) phthalate-d_{38} (DEHP-d_{38}), n-hexane-d_{14}, and n-pentane-d_{12} were obtained from C/D/N Isotopes Incorporated. (Pointe-Claire, Quebec, Canada).

4.2.4 Subject Enrollment

The parents of thirty children aged 2-9 with autism or pervasive developmental disorder-not otherwise specified (PDD-NOS) reviewed the inclusion and exclusion criteria with a research coordinator. Following the policies outlined in the Common Rule of Responsible Conduct of Research in Humans and the Declaration of Helsinki, parent consent was attained and child assent was gained whenever possible. A psychologist with certification in autism diagnostic observation schedule (ADOS) met with each child and a diagnosis of autism or PDD-NOS allowed participation in the study. Each child was paired by age, gender, and household income with a control child recruited by a flyer approved by the IRB. Following consent and child assent, control children underwent a history and physical examination to rule out genetic, developmental, neuropsychiatric disorders, and an immediate family member with ASD. Control children then underwent identical ADOS evaluation, which confirmed the absence of autism.

4.2.5 Blood Draws

Approximately 30 mL of blood was drawn at the child's home and processed for storage within two hours. The blood was centrifuged and aliquots were separated into serum, plasma, and red blood cells, before storage at −80 °C. All blood processing and analysis was performed in a laboratory certified as an International Organization for Standardization Class-5 cleanroom. The serum was processed from whole blood that was drawn using tubes specifically certified free of volatile organic compounds.

4.2.6 Behavioral Rating Scales and Observations

Behavioral rating scales were filled out by a parent or guardian. The rating scales included the social communication questionnaire (SCQ), aberrant behavior checklist (ABC), Gilliam autism rating scale (GARS), autism treatment evaluation checklist (ATEC), PDD behavior inventory (PDDBI), and childhood autism rating scale (CARS). For analytical comparisons, three ADOS observational results, including the communication and social domain values, as well as the total ADOS score (a sum of the communication and social domain scores) were determined at The Children's Institute of Pittsburgh by a clinician with research level certification in ADOS performance. [27-32]

The ADOS is a widely accepted standard measure of deficits associated with ASDs. [33] Nomenclature at the time of the study supported a total score of 7 indicating the presence of ASD. Research on the development of the ADOS found that individuals categorized in the most behaviorally severe subset (termed "lower-autism") had a minimum total ADOS score of 14. [33] Therefore, this study utilized these ADOS-suggested categorizations, with a total score of 7 indicating the presence of ASD and a total score of 14 differentiating individuals into the most behaviorally severe subset for data processing. This study's data was accumulated prior to the publication of Diagnostic and Statistical Manual V.

4.2.7 Validation and Statistical Assessment

Means and standard deviations were calculated using five replicate analyses. The lower limit of quantitation (LOQ) was calculated as ten times the standard deviation of repetitive measurements of the blank. All statistical comparisons were performed at the 95% confidence interval (CI). Variance (σ^2) was assessed as the square of standard deviation. Accuracy was

evaluated by determining percent error from a certified value. For cohort comparisons, a standard p-value cutoff was set as 0.05.

Validation was performed by spiking certified standards for each compound into blank-subtracted blood serum and analyzing by the above method. Experimental concentrations were statistically compared with certified values.

4.2.8 Mean Xenobiotic Body-Burden and Exclusion Criteria

Concentrations of all xenobiotics quantified in an individual were pooled into one variable, termed mean xenobiotic body-burden (MXB), for each individual. MXB was calculated as the simple mean of all quantifiable analytes of interest for each individual. Usage of the unweighted mean was chosen to reduce the risk of biasing MXB toward a particular compound or chemical class. The MXB value of an individual averaged only those xenobiotics that were quantified above the compound-specific LOQ. Following peer-reviewed methods for handling missing variables in pooled data, individuals possessing quantifiable concentrations in <50% of the studied xenobiotics were excluded from the MXB comparative analyses. [34]

4.2.9 Linear and Logistic Regressions

Linear regression was performed on the separate cohorts to determine the relationship between total ADOS score and MXB. Logistic regression was performed and all probabilities were reported in decimal form. A peer-reviewed method for adjusting logistic regression for rare-event data was used. [35] In this way, adjusted regressions were produced assuming 0.5%, 1%, 2%, or 3% national prevalence of the most severe autism diagnosis.

4.3 Results and Findings

As described in Chapter 3, the analytical method was validated using blank-subtracted serum spiked with certified concentrations of each studied xenobiotic prior to this study to assess accuracy, precision, and limits of detection and quantitation. Full validation and LOQ data can be found in Chapter 3. For all results and discussions, children with ASD are referred to as the cohort with ASD or ASD cohort (n=30) and matched controls as the control cohort or controls (n=30).

4.3.1 Qualitative Assessment of Exposure

All participants displayed unique patterns of non-metabolized xenobiotics in serum. Many compounds were detected at levels below the analyte-specific LOQ but above the 3-to-1 signal-to-noise limit of detection.

Detection results can be found in figure 4.1, showing percentages of children with detectable amounts of each specified xenobiotic in their serum and distribution of detection rates between cohorts. Perchloroethylene, n-hexane, benzene, and toluene, were detected in >80% of all children tested. Acetochlor, metolachlor, and pendimethalin were detected in >90% of the children in the study. PCBs 153, 138, 52, 28, and 101 were detected in 50%, 17%, 14%, 12%, and 9% of all children in the study, respectively.

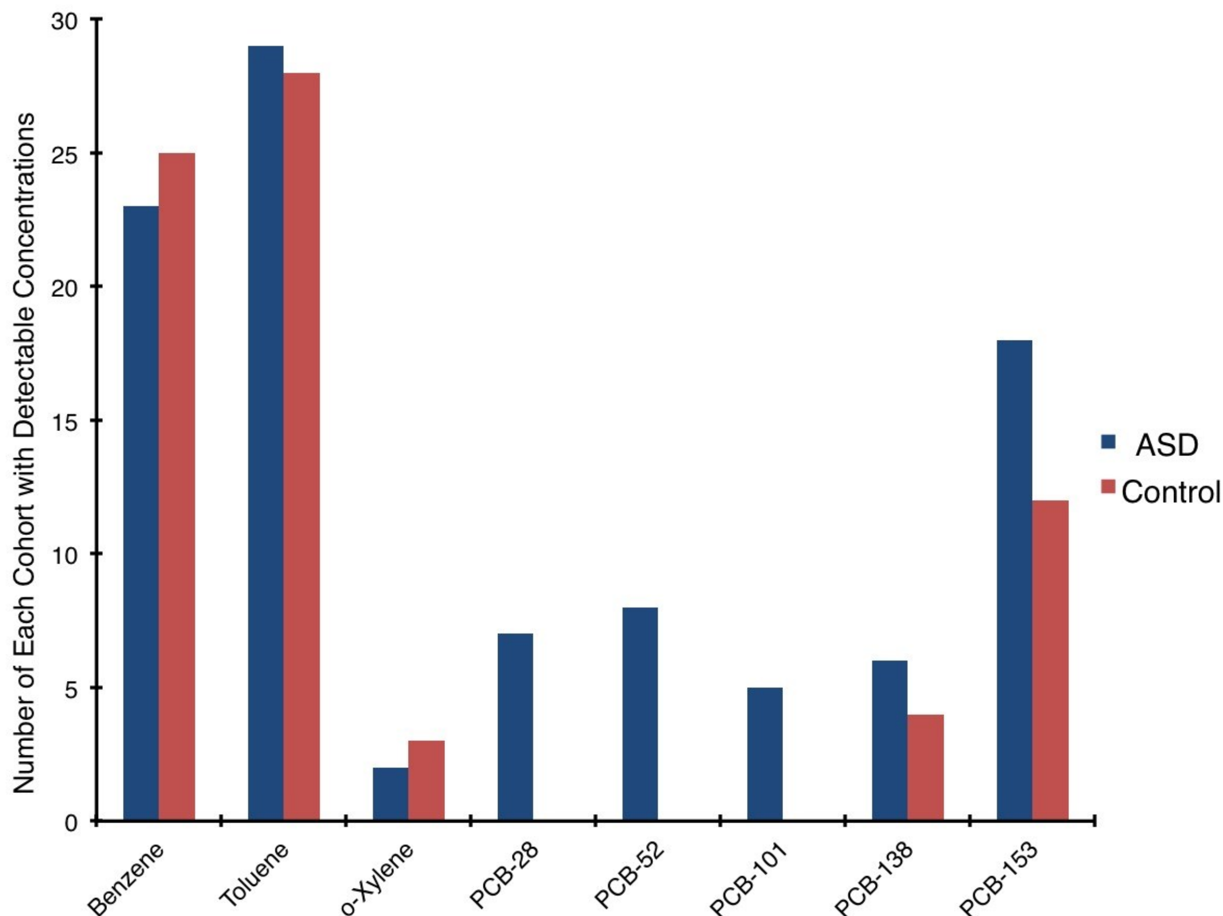

Figure 4.1: Percentage of the total population displaying detectable amounts of each compound, showing portion of compound detected in each cohort

PCBs 28, 52, and 101 were detected in only the ASD cohort. PCBs 138 and 153 were detected in 2.5-times and 2-times, respectively, more of the ASD cohort than controls. The pesticides acetochlor, metolachlor, and pendimethalin were detected in a greater number of controls than the cohort with ASD, 20%, 8%, and 4% greater, respectively. Overall, the cohort with ASD contained a greater number of children with detectable levels of PCB and the control cohort contained a great number of children with detectable levels of pesticides common to Western Pennsylvania.

4.3.2 Quantitative Cohort Comparisons

Full quantitative results and statistical tests for each xenobiotic by cohort can be found in table 4.1. The pesticide metolachlor was quantified at a higher mean concentration in the control cohort than the cohort with ASD (p=0.021). The pesticide pendimethalin trended toward higher concentrations in the control cohort, outside of statistical significance (p=0.118). No other significant differences were discovered between the two cohorts for any specific xenobiotic.

Table 4.1: Full quantitative results by cohort for all xenobiotics included in the study

	ASD Cohort (μg/g)		Control Cohort (μg/g)		
	n	Mean (95% CI)	n	Mean (95% CI)	p-value*
Benzene	4	0.263 (±0.13)	6	0.291 (±0.087)	0.652
Toluene	26	0.143 (±0.10)	28	3.32×10^{-2} (±1.34)	0.123
o-Xylene	3	1.35×10^{-3} (±1.2)	0	-	-
Hexane	24	11.7 (±2.3)	29	9.44 (±0.81)	0.684
PCE	24	6.38×10^{-2} (±1.8)	27	5.64×10^{-2} (±2.5)	0.614
PBDE-47	1	0.00208 (N/A)	4	2.18×10^{-3} (±1.1)	-
PBDE-99	4	7.19×10^{-3} (±5.0)	3	1.41×10^{-2} (±0.89)	0.0987
Chlorpyrifos	10	2.27×10^{-4} (±1.1)	12	1.75×10^{-4} (±0.30)	0.891
Pendimethalin	28	1.07×10^{-2} (±1.01)	29	2.01×10^{-2} (±5.4)	0.118
Metolachlor	28	5.18×10^{-3} (±2.8)	30	1.20×10^{-2} (±0.51)	0.021
Acetochlor	25	0.182 (±0.018)	30	0.160 (±0.026)	0.695
DEHP	19	3.95 (±1.7)	19	3.29 (±1.01)	0.697
Pentane	0	-	0	-	-
Pooled Mean	9	1.36 (±3.0)	9	1.21 (±2.4)	0.846

- Not enough replicates for calculation

* Comparing concentration from ASD cohort to control cohort

Comparing the pooled mean of all compounds from the ASD cohort to the pooled mean for all compounds in the control cohort yielded no significant difference (p=0.846). O-xylene, PBDE-47, and PBDE-99 were quantifiable in <5 individuals in each cohort and were not included in data pooling statistics to eliminate bias toward any specific compound, leaving nine xenobiotics with which to compare quantitative variance.

The ASD cohort and controls both demonstrated significantly higher concentrations of benzene, toluene, and DEHP (all $p<0.0001$), compared with concentrations found in a recent national study. [36] Figure 4.2 shows a comparison between geometric mean concentrations observed in a recent national study and statistical mean concentrations observed in this study for the two cohorts.

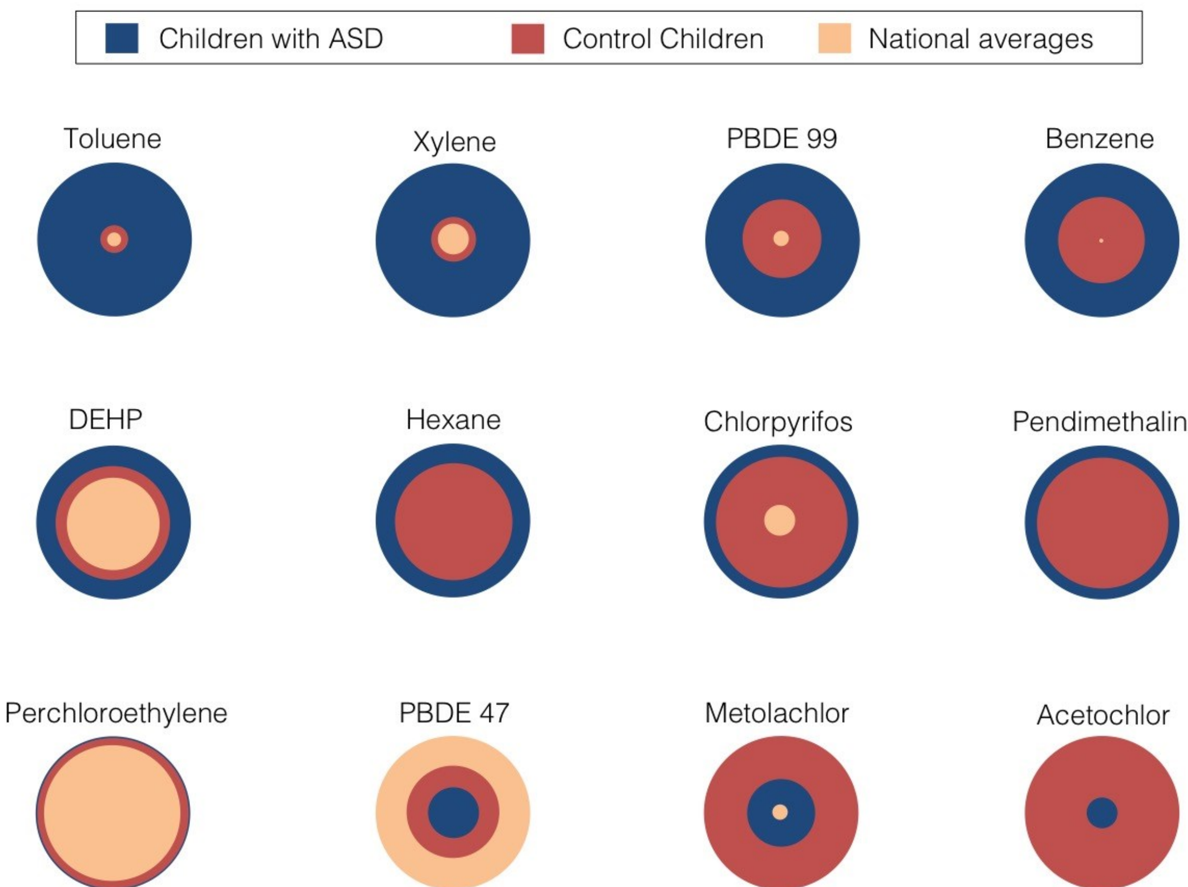

Figure 4.2: A comparison between geometric mean national average concentrations and the statistical mean concentrations observed for the cohorts in this study

A quantitative comparison between the two cohorts in this study with national study averages for children of similar ages yields significant differences. Figure 4.3 demonstrates the comparison between the three populations on a logarithmic scale. The children living in a similar

geographic region (Pittsburgh, PA) demonstrated similar mean concentrations to each other, but differed dramatically from the national averages for the selected compounds. Confidence intervals were not included in this analysis due to the extremely wide range of concentrations found within each population. The cohorts from this study demonstrated significantly greater levels of benzene, toluene, and DEHP compared with the children in national studies.

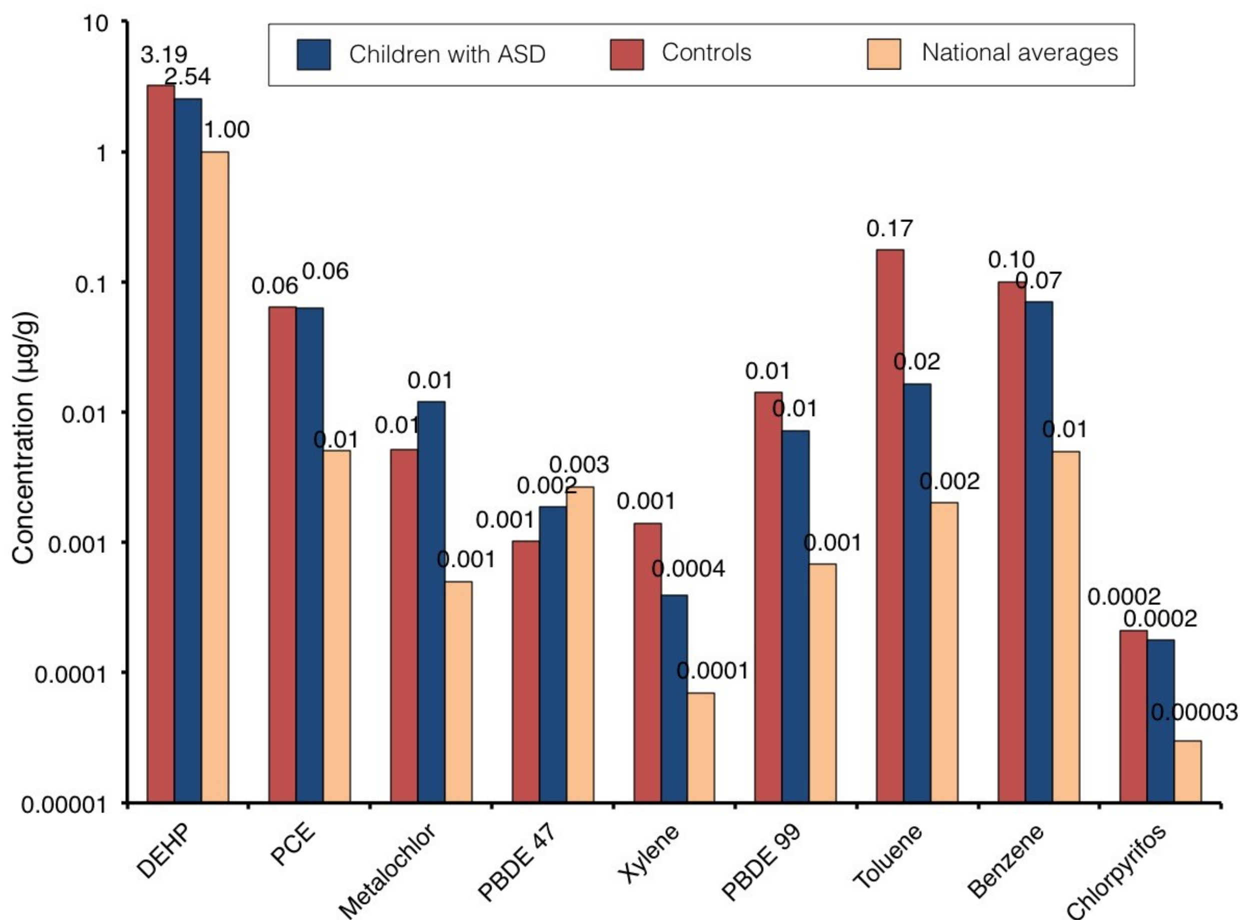

Figure 4.3: Comparison between the two cohorts from this study and concentration values from recent national study, without confidence intervals

4.3.3 Quantitative Variance

Detailed variance and relative standard deviation (%RSD) data can be found in table 2. Overall, the cohort with ASD demonstrated a greater range of concentrations for most of the

xenobiotics studied compared with controls. The cohort with ASD displayed significantly greater variance in the pooled mean of all compounds compared with controls (p=0.006) and greater %RSD for seven of the nine xenobiotics included in the variance analysis.

Table 4.2: Quantitative variance and relative standard deviation for each xenobiotic and pooled mean by cohort

	ASD Cohort		Control Cohort	
	Variance (σ^2)	%RSD	Variance (σ^2)	%RSD
Benzene	0.0089	36	0.0075	30
Toluene	0.085	170	0.0025	100
Xylene	5.9×10^{-5}	57	-	-
Hexane	29	46	3.9	21
PCE	0.0017	65	0.0040	110
PBDE47	-	-	2.3×10^{-7}	33
PBDE99	1.3×10^{-5}	50	3.1×10^{-5}	39
Chlorpyrifos	2.5×10^{-8}	69	2.2×10^{-9}	267
Pendimethalin	0.00079	260	0.020	700
Metolachlor	5.3×10^{-5}	140	0.00020	120
Acetachlor	0.0018	23	0.0054	46
DEHP	11	85	4.7	66
Total Mean	4.1	290	0.87	260

4.3.4 Mean Xenobiotic Body-Burden and Behavioral Severity

All individuals in the ASD cohort scored 7 or higher on total ADOS, meeting the cutoff for ASD diagnosis. All individuals in the control cohort achieved a score of 6 or less. The difference in mean scores of the two cohorts on each of the rating scales and observations used in this study was highly significant. [37] No significant correlations were observed between specific xenobiotic concentrations, MXB, or rating scale performance with age, ethnicity, sex, or socio-economic status, indicating matched-pairing was statistically irrelevant.

The exclusion criteria for MXB eliminated nine from the ASD cohort and six controls from this analysis due to insufficient data. Total ADOS performance as a function of MXB for each individual produced two disparate trends, demonstrated in figure 4.4. The ASD cohort

(n=21) exhibited a linear trend between increasing MXB and total ADOS performance (r=0.5384, p=0.011), while the control cohort (n=24) demonstrated no linear trend or correlation with MXB (r=0.1121, p=0.60). MXB also significantly correlated with the two ADOS sub-domains: communication (p=0.020) and social (p=0.050) in the cohort with ASD, but not controls (p=0.80, p=0.34, respectively). The Pearson correlation, r, is used as a measure of linear relationship, expressed as a value between -1 and 1. While there is no agreed-upon limit for r-value significance, researchers have implied that correlations as low as 0.3 demonstrate statistical significance.[38]

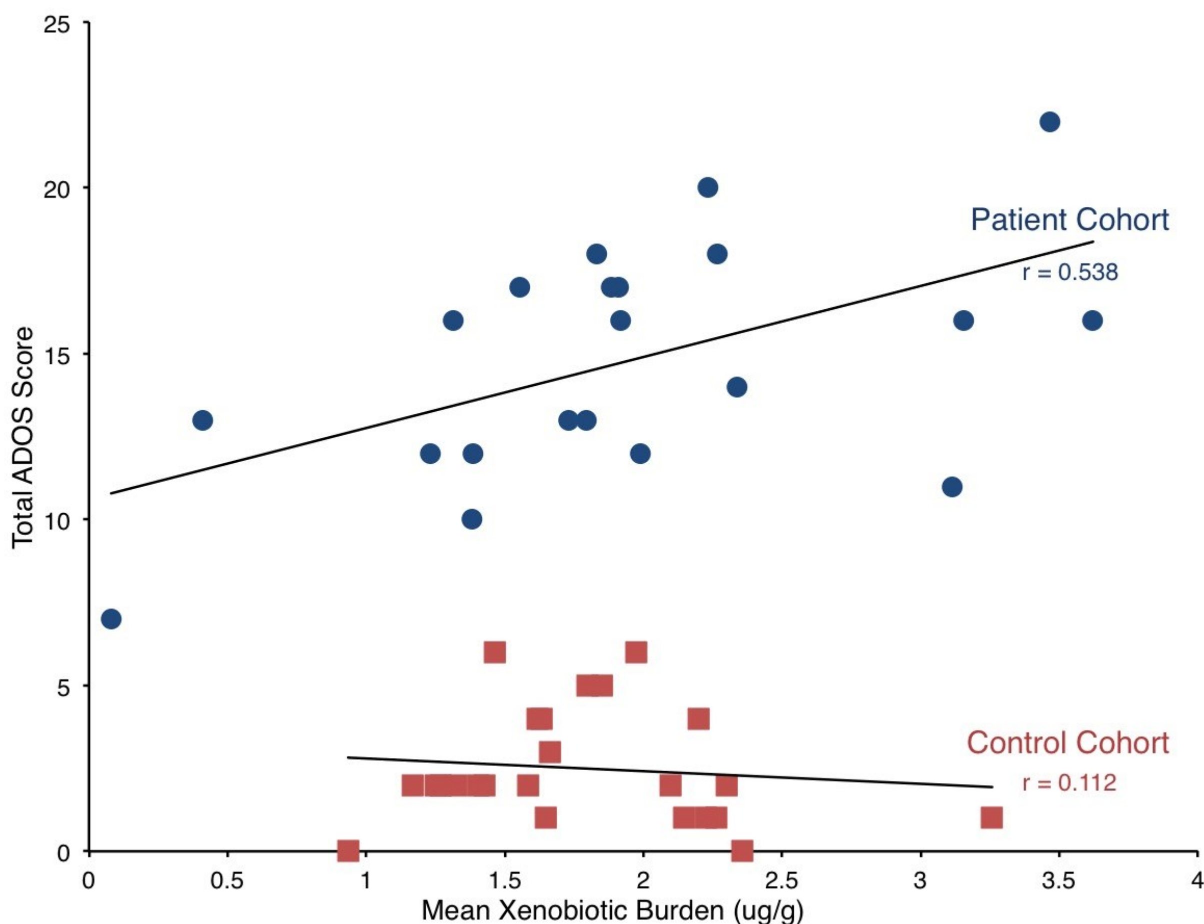

Figure 4.4: Individual total ADOS performance as a function of mean xenobiotic burden in serum for ASD cohort and controls, showing the compound class with the greatest relative contribution to mean

No other parent questionnaires or rating scales produced significant correlations with MXB in either cohort. Hexane, PCE, DEHP, and chlorpyrifos concentrations demonstrated the most significant linear correlations with MXB (p=0.001, 0.008, 0.02, 0.03, respectively). No individual xenobiotics correlated significantly with total ADOS or ADOS sub-domains.

This experiment was not designed with a large enough sample size, determined by a power analysis (p=0.80), to accurately determine if mean MXB of the whole ASD cohort differed significantly from the mean MXB of the total study population. For this type of analysis, a minimum sample size of 600 would be required, given the mean population MXB, mean ASD cohort MXB, and standard deviation of the population. Mean MXB of the ASD cohort did not differ significantly from mean MXB of the controls (p=0.491). Within the ASD cohort, mean MXB of individuals with total ADOS ≤14 (n=10) were nearly significantly lower (p=0.054) from those with total ADOS >14 (n=11). Mean MXB of individuals with total ADOS >14 were significantly greater than the control cohort (p=0.029) and the rest of the ADOS ≤14 study-population (n=34, p=0.018), as summarized in figure 4.5.

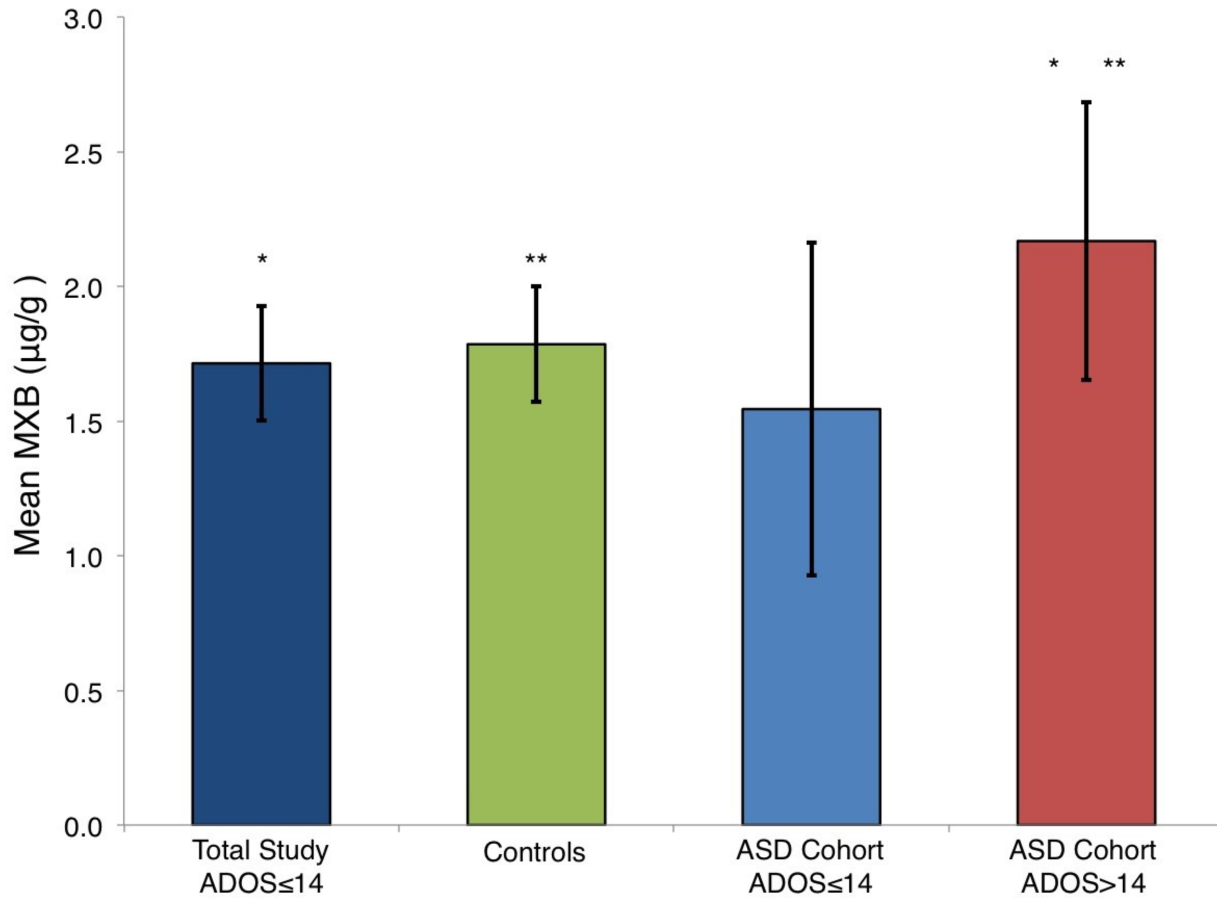

Figure 4.5: Comparisons of MXB between differing subgroups, with 95% CI
*, ** Pairs of significantly different means

A logistic regression was performed with four rare-event adjustments, shown in figure 4.6. Using total sample size (n=45), MXB was found to be a predictor variable for probability of obtaining a total ADOS >14 (O.R. =3.43 [1.14, 10.4]) with a statistically significant overall fit (p=0.0287, coefficient: 1.23). Adjustments were performed using assumed national prevalence rates of behaviorally severe autism of 0.5%, 1%, 2%, and 3%. These adjustments do not alter the O.R. or overall significance of model fit.

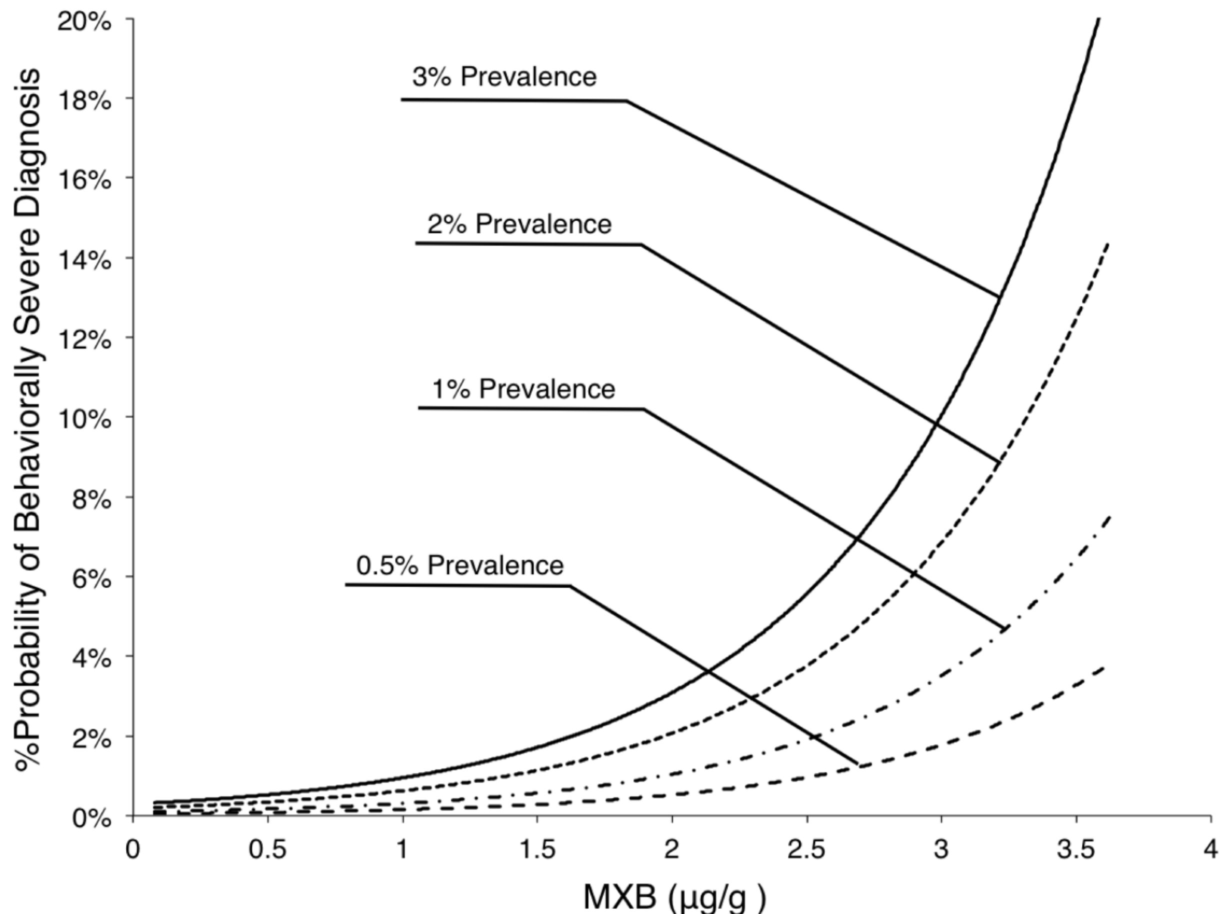

Figure 4.6: Logistic regression showing MXB as a predictive variable for diagnosis in the most behaviorally severe ADOS classification (total ADOS >14), incorporating adjusted intercept values for assumed national prevalence of the most behaviorally severe ADOS classification.

4.4 Discussion[1]

The purpose of this cohort study was to evaluate the differential relationship between concentrations of organic pollutants and behavioral severity in children with ASD and matched controls. This study was conducted in association with a broader study also evaluating elemental and immunological markers. [37, 39] Researchers have suggested that children with ASD may have dysfunctional cellular-level pathways necessary for removal of xenobiotics from the body,

[1] Discussions and conclusions for this work were produced in association with The Children's Institute of Pittsburgh and represent a collaborative viewpoint citing, where opinion was not the sole work of this author, either peer-reviewed literature or the consulting physician associated with this IRB-approved study.

though concerns have been expressed over the design and methodologies of some studies. [40] The work discussed here addressed many of these concerns by measuring environmentally relevant serum-concentrations and used matched controls to investigate relationships between and among organic pollutants and autism rating scales/observations. Compared with a recent national study, both cohorts demonstrated significantly elevated concentrations of benzene, toluene, and DEHP, indicating that serum-concentrations of many of the studied organic pollutants may vary based on location.[36]

The hypothesized existence of a physiological/environmental interaction mechanism contributing to the behavioral phenotype of autism in susceptible individuals cannot be rejected by this research. Three findings provide support for this hypothesis: quantitative and qualitative differences in specific organic pollutants, significantly different quantitative variance, and significantly altered levels of methylcobalamin (MeCbl) in the two cohorts. Evidence of genetic predisposition or susceptibility was suggested by finding a differential neuro-behavioral response to MXB between the two cohorts. These findings and their relations to the hypothesized pathophysiology are explored further.

4.4.1 Detoxification and Methylation Efficacy

Observed neurobehavioral and biochemical differences between the cohorts may be explained by differential functioning of cellular detoxification pathways that may have led to or exacerbated central nervous system excitotoxicity, a feature found in autism. [15] The control cohort displayed a significantly greater serum-concentration of metolachlor, a common pesticide in Western Pennsylvania. Presuming that children with ASD and controls were exposed to relatively similar regional environments, the children with ASD may have had greater difficulty

in moving metolachlor from cells and tissues into the blood. Qualitatively, all PCBs were detected in a greater percentage of the ASD cohort than controls, suggesting the children with ASD may have had greater difficulty in metabolizing PCB compounds, removing them from serum. Previous studies have noted that elevated PCBs can increase intraneuronal calcium and alter calcium signaling via changes in the ryanodine receptor, which can upset the balance between glutamate and GABA neurotransmission, creating central nervous system excitotoxicity. [12]

Researchers associated with the current study have found significantly greater levels of methylmalonic acid (MMA) in the cohort with ASD compared with controls. [37] Increased MMA indicates deficiency in MeCbl, a form of vitamin-B_{12}, contributing to impaired MS function. [41] Impaired MS function may have disrupted multiple pathways associated with both detoxification and methylation in the children with ASD, including dopamine-stimulated PLM necessary for neuronal synchronization and normal functioning of the central nervous system.

With the exception of metolachlor, no other concentration differences were observed between the cohorts for specific xenobiotics. However, the ASD cohort displayed significantly greater variance around the mean of individual organic pollutants, as well as the pooled mean, compared with controls. This difference in variance further suggests the children with ASD may have had greater variability in detoxification efficacy compared with controls. The suggested link between methylation, detoxification, and neuropathology is supported by recent research finding that brains of individuals with ASD display significantly greater variability in neuronal synchronization compared with controls. [22]

4.4.2 Genetic Predisposition or Susceptibility

Differing neuro-behavioral trends in relation to MXB were observed in the two cohorts. The significant correlation between MXB and total ADOS score in the ASD cohort, but not controls, is a fundamental expectation from the hypothesis of genetic predisposition for susceptibility to environmental triggers.[13] As demonstrated in tables 4.3a and 4.3b, correlations were observed in neither cohort between mean concentration of specific pollutant and total ADOS score. However, correlations were observed between MXB and hexane, perchloroethylene, chlorpyrofos, and DEHP. These correlations between increasing concentration of specific pollutants and increasing MXB indicates that these four compounds were the primary influence on MXB values, though the four compounds themselves suggested no correlative trend with autism severity.

Table 4.3a: Correlations between xenobiotics and MXB and total ADOS for the ASD cohort

	Correlation with MXB		Correlation with total ADOS	
	r-value	p-value	r-value	p-value
Hexane	0.77	<0.001	0.09	0.69
PCE	0.55	0.008	-0.11	0.61
DEHP	0.51	0.02	0.023	0.93
Chlorpyrifos	0.46	0.032	0.29	0.44
Metolachlor	-0.31	0.16	0.041	0.84
Pendimethalin	-0.16	0.48	0.15	0.43
Toluene	-0.04	0.85	0.16	0.54
Acetachlor	0.088	0.7	0.012	0.95
Benzene	-	-	-	-
Xylene	-	-	-	-
PBDE47	-	-	-	-
PBDE99	-	-	-	-

Table 4.3b: Correlations between xenobiotics and MXB and total ADOS for the control cohort

	Correlation with MXB		Correlation with total ADOS	
	r-value	p-value	r-value	p-value
Hexane	0.57	0.003	-0.19	0.32
PCE	0.399	0.053	-0.21	0.31
DEHP	0.43	0.03	-0.06	0.83
Chlorpyrifos	-0.42	0.036	-0.34	0.27
Metolachlor	-0.02	0.9	-0.012	0.94
Pendimethalin	0.12	0.56	-0.27	0.15
Toluene	0.133	0.53	-0.18	0.49
Acetachlor	0.3	0.14	-0.18	0.34
Benzene	-	-	-	-
Xylene	-	-	-	-
PBDE47	-	-	-	-
PBDE99	-	-	-	-

- Not enough data for correlation calculations

While logistic regression across the study population yielded a significant model using MXB as a predictor variable for the most severe behavioral classification, disagreement exists on the national-prevalence of diagnosis. Most studies have reported ASD prevalence-rates of 1 – 2%, [42] but robust research has not been conducted on prevalence of the classification of "lower autism." Given this uncertainty, four rare-event adjustments were used: 0.5%, 1%, 2%, and 3% national prevalence, found in figure 4.6.[43] These logistic regression results imply that for every 1 μg/g increase in MXB, the probability that an individual would present an ADOS score >14 increased by a factor of 3.4.

The results from this study support the hypothesis that ASD pathophysiology may relate to interactions between physiology and the chemical environment in genetically susceptible individuals. However, direct studies of detoxification and methylation systems, along with neuropathology, and their relationship to chemical xenobiotic levels, are necessary in future studies. While this study was not designed to use organic pollutant concentrations for medically diagnostic purposes, these statistically significant results suggest the presence of a cellular-level

mechanism governing the varying degrees of interaction between physiology and the chemical environment in children with ASD and controls.

4.5 Conclusion

This study lends further support to the growing body of literature that relates ASD diagnosis with exposure to environmental pollutants. With the exception of metolachlor, results indicated the cohorts had statistically similar concentrations of individual organic pollutants studied; however, the ASD cohort demonstrated a significantly greater range of concentrations. The results from this study were unique in finding the mean xenobiotic burden of organic pollutant concentrations to significantly correlate with behavioral severity in children with ASD, but not controls. The mean xenobiotic burden also significantly correlated with increased risk of the most behaviorally severe ASD diagnosis across both cohorts with an odds ratio of 3.4. These results support the hypothesis that a portion of the pathophysiology leading to autism may be attributable to exposure to organic chemical pollutants. Studies that relate copy number variant data from oligoarrays, whole exome sequencing, and whole genomic sequencing to efficacy of methylation and detoxification processes and behavioral phenotype are reasonable next steps in the evaluation of the hypothesis that environmental triggers contribute to the etiology and maintenance of autism spectrum disorders.

4.6 Limitation

This work was not intended to, nor do the results, support the hypothesis that the studied cohorts were exposed to greater amounts of environmental pollutants than other populations. Likewise, these results do not differentiate between causation and correlation in relation to the

MXB vs. ADOS findings. The correlation observed in the children with ASD may be a symptom of a malfunctioning detoxification system or may be more causative in nature. Future studies should investigate the nature of this relationship. Few studies have quantified non-metabolized markers of environmental exposure in human blood; therefore, accurate and nationally relevant blood-concentration data was unavailable for many compounds, specifically chlorinated pesticides.

Blood serum was the only bodily fluid used for extraction and quantification in this study. While serum was not used as a proxy for adipose tissue or other fluid, the experimental variability in serum extractions is well known. To address this inherent variability, SBSE and IDMS were used, as these methods have been reported in literature as highly reproducible between and among laboratories. However, standard laboratory practice of extraction of lipophilic compounds, such as pesticides and PCBs, recommends correcting and standardizing for the lipid content in the blood of an individual. Lipid correction has been performed in numerous peer-reviewed and government studies; however, simulation studies have found that correcting for lipid content has high probability to introduce bias into quantification. In this study, lipid correction was not performed due to the limited volume of serum available from each child. Novel analytical methods were developed and optimized to use sample volumes significantly smaller than typically found in literature, which eliminated the possibility of total lipids analysis.

The simplistic calculation of MXB has resulted in a variable which should not be viewed as the only statistical comparison between ADOS and organic pollutant concentration. While table 4.3a and 4.3b show that no one compound drove the observed increase in MXB, nor did any single compound correlate with ADOS increase in the children with autism either, it must be

noted that future analysis of this dataset (as well as future studies) will employ the use of discriminant analysis and principle component analysis to ascertain any internal structure that may exist inside of the MXB and ADOS datasets. Likewise, future experimental design may draw upon the MXB values obtained in this study to decide upon appropriate sample size, given the power with which researchers hope to discriminate data.

4.7 References

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Chapter 5: Conclusions and Future Research Directions

5.1 Environmental Health Conclusions

The research described within this document produced two novel methods for the analysis of organic pollutants in blood serum, applicable to environmental analysis. The first method significantly improved upon existing industry accepted methods for the analysis of pollutants in serum, while utilizing commonplace methods for organic analyte extraction, SPME.[1] This SPME method was flawed, however, in that immersive extraction was not possible, contrary to multiple existing peer-reviewed methods present in respected journals. Regardless of the data in these references, immersive analysis using SPME could not be replicated in this laboratory. This optimized and validated SPME-IDMS method was the first method of its kind to correct the inherent variability present in SPME analysis by using the quantitative methodologies described in EPA Method 6800.[2]

The second method produced by the work described in this document utilized SBSE as the primary mode of organic pollutant extraction from blood serum and environmental water samples. As a technology, SBSE has recently gained wider acceptance in the fields of water and food analysis, but has not been extensively research for clinical or biological research. The SBSE method produced in this work, when combined with IDMS, resulted in a significantly superior method when compared with current industry accepted methods for extraction and quantification. This S-SBSE-IDMS method was the first of its kind to effectively recover volatile, semi-volatile, and nonvolatile compounds simultaneously from one sample using one stir-bar with no additional sample modifications.[3] The single stir-bar technique provided additional opportunity for lengthy stir-bar extractions and irreproducible analyte recovery;

however, when combined with IDMS as described in EPA Method 6800, many common error-introducing analytical processes were eliminated.

The developed and optimized S-SBSE-IDMS method was applied to collaborative work focused on the impact of environmental pollutants on human health.[4] The results of this application have provided invaluable contributions to the fields of exposomics, environmental human health, and pathophysiological research on autism spectrum disorders. The high quality of the data produced using the described S-SBSE-IDMS method would be difficult to replicate with currently accepted techniques for either extraction or quantification. The analytical quality afforded by SBSE and IDMS have pushed both sensitivity and selectivity to a point at which environmentally relevant, actionable results can be acquired.

The sourcing and identification of exposure routes for the environmental pollutants found in the children with ASD must be a significant future research goal. Potential exposure routes include municipal water, ground water, soil, air, and indoor environment, among many others. For each of these sampling requirements, unique challenges will be discovered that require unique but effective solutions. For the standard analysis of wastewater and drinking water, the developed S-SBSE-IDMS method was successfully applied to unknown samples supplied to this laboratory as part of a working group to report on and recommend updates to EPA Method 625. This reported S-SBSE-IDMS method produced data among the highest quality of all 23 participating laboratories. All recommendations on the update to Method 625 will appear in a peer-reviewed publication based upon the method developed for environmental water analysis presented in Chapter 3.

5.2 Method Transfer Conclusions

The methods developed in Chapter 2 and 3 presented feasible solutions to the problems of method transfer cited in their respective Chapters. Many published analytical methods for biological analysis have been reported as difficult to replicate in independent laboratories by analysts or technicians. One of the specific aims of the work described in this document was the elimination of complex, error introducing sample preparation steps. The results presented in Chapter 3 suggest that this objective was achieved and the hypothesis that IDMS can be used to eliminate the necessity for analytical laboratory expertise from all analysts and technicians has been supported as well.

Much of the quantitative error introduced into sample analysis can be attributed to imprecise sample preparation. Spiking of isotopes for IDMS prior to all other sample preparation steps creates a scenario where many analytical steps (dilution volumes, extraction times, extraction temperatures, etc.) can be imprecise without affecting overall analytical quality, as the ratio between isotopically labeled and naturally occurring compound in said sample will not be altered. The results presented in Chapter 3 demonstrate that a highly transferable analytical method has been created that reduces the influence of analyst skill on overall analytical quality.

5.3 Future Research Outlook for Method Transfer

Future research is required to produce a standard method for the analysis of blood serum beyond a strictly academic setting. As an example, analytical methods for the analysis of environmental samples have been standardized for decades by the EPA. These environmental methods have been designed to produce similar values regardless of the analyst or laboratory producing the quantitative results. Biochemical measurements must be standardized in a similar

manner. The ability for any moderately trained analyst to produce high quality analytical data and similar values compared with the expert analyst is a necessity in this age of increasingly personalized medical care.

Appendix A described several additional experiments performed to further improve the quality of analytical method transfer and reduce the time and cost of implementing the S-SBSE-IDMS method developed in Chapter 3. The first experiment described in Appendix A focused on an alternative to venous blood draws from the sample collection process for the quantification of organic pollutants in blood. The second set of experiments sought to remove the consumable-heavy liquid nitrogen cryo-trap process from sample analysis. The final section of Appendix A presents the hypothesis that IDMS may be used to pre-calibrate SPE columns for the analysis of biological and environmental samples to remove the isotope spiking procedure from the in-laboratory procedure. These experiments have produced promising results, yet further research will be necessary to increase validity and robustness.

Venous blood draws can be costly and can introduce unnecessary risk of infection and biological hazard into laboratory research. More hazardous still, an undergraduate teaching laboratory would find it exceedingly difficult to gain approval for the analysis of venous blood in an experimental procedure. Yet, increasingly, recent graduates are gaining employment in clinical and commercial laboratories where they are expected to handle and analyze blood and other biological fluids. It was the objective of section A.2 to design an undergraduate teaching laboratory to minimize the cost and hazards associated with analysis of blood. This objective was accomplished using the S-SBSE-IDMS method developed in Chapter 3 by allowing students to analyze microliters their own blood obtained from finger-stick devices. While students reported a higher investment in learning outcome through analysis of their own blood, further research is

necessary on the full validation of the finger-stick collection method.[5] Particular focus must be given to the stability of volatile analyte in blood when exposed to ambient air prior to collection via micropipette. In venous blood collection, blood is transferred immediately from vein to vacuum-sealed container without ever encountering ambient air. A schematic of the proposed finger-stick method is provided in figure 5.1. This must be thoroughly researched before a research-oriented publication is submitted.

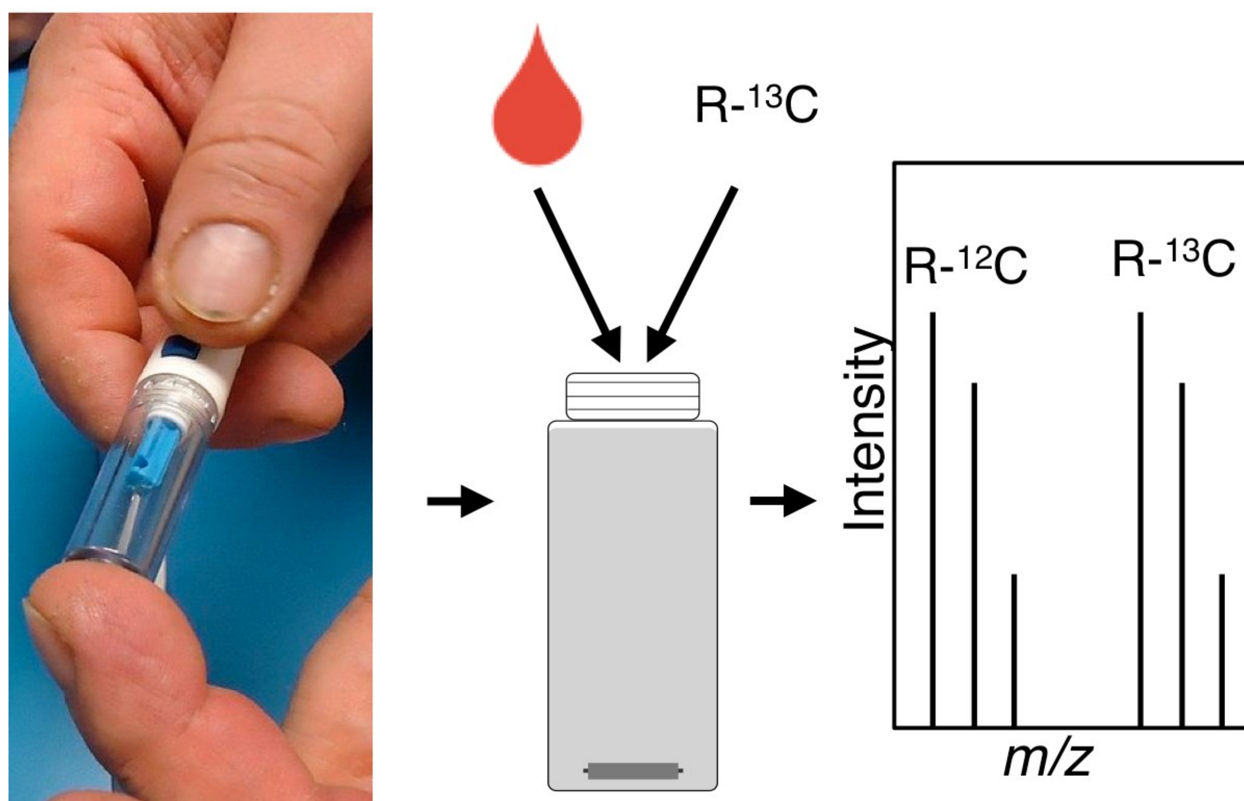

Figure 5.1: Schematic of the finger-stick procedure to produce direct analysis of microliters of whole blood

Trapping of volatilized compounds with liquid nitrogen has been the standard with SBSE, and SPME before it, since its creation. However, one SBSE-GC/MS system under high-use conditions may consume up to 180 liters of liquid nitrogen per week. This is a significant consumable. Work was performed to remove the requirement for temperatures associated with

liquid nitrogen using adsorbent polymers in the GC inlet. The hypothesis of this research was that adsorbent polymers, contrary to inert surfaces like quartz or glass wool, would provide a second trapping mechanism and allow the use of higher temperatures. If effective trapping could occur at approximately 0 °C, then thermoelectric cooling could be used instead. The advantage of thermoelectric cooling is the significant reduction in consumable waste and the high power efficiency. This hypothesis was supported by research presented in Section A.3.2, but further work is necessary to validate these findings. Proper method validation must be performed to ensure that precise and accurate measurements can be made at these new trapping temperatures with the new polymer traps. Additionally, work must be performed to ensure that complete analyte desorption is taking place. While no carry over was observed, irreversible compounds retention by either the SBSE or inlet liner must be investigated.

The final set of experiments presented in Appendix A hypothesized that traditional SPE columns could be pre-loaded with isotopes to remove the isotope spiking procedure from the hands of in-laboratory analysts, effectively transferring the skill of the expert analyst into outside laboratories.[6] This hypothesis was tested using both environmental and toxicological samples and analytes. While stable quantification was achieved for pesticides in groundwater and illicit drugs in synthetic urine, further work must be performed to increase on-column stability of isotopes, possibly by forming a complete seal on the SPE column during storage to prevent drying. A schematic of the pre-loading process and analytical workflow is provided in figure 5.2.

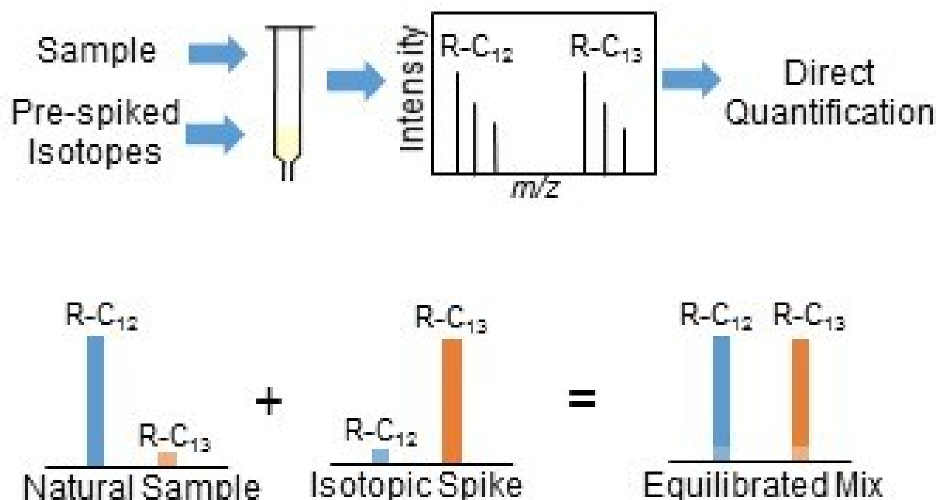

Figure 5.2: Broad schematic of total analytical procedure

Further exploration of these hypotheses may result in the generation of a high-quality analytical method capable of seamless transfer between and among laboratories and analysts.

5.4 Future Research Outlook for Theoretical SBSE Investigations

Appendix B presents findings on the investigation of the mechanisms governing SBSE. Specifically, an investigation was performed to differentiate between a primarily surface-adsorption extraction and a bulk-PDMS volume based extraction. Theoretical models on analyte recovery predicted two scenarios: either the extraction was volume based or the analyte recovery would increase with increasing surface area. Unfortunately, analyte recovery decreased with increasing surface area. These results were unexpected. Several theories are proposed and discussed in Appendix B, but the most reasonable is that stir-bar extraction contains elements of bulk PDMS shielding an analyte from interaction with the solvent to prevent back-extraction. This work has much research remaining, however. First, one should utilize a direct mass spectrometric technique to determine the exact depth of penetration of commonly analyzed molecules, and how this depth may change based on K_{ow} or volatility. A laser-ablation triple

quadrupole system may be able to scan along the diameter of a stir-bar cross section following sample extraction to determine, down to a nanometer, the exact depth of penetration of common analytes. Secondly, the findings from Appendix B must be replicated using different compounds to determine the influence of chemical characteristics like analyte structure, functional class, volatility, molecular weight, and many others.

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Appendix A: Optimizations to Improve Method Transfer

A.1 Introduction

As a primary focus of the research described here within, valid inter-laboratory method transfer requires that analysts, regardless of individual analyst skill, reproduce an analytical method with similar analytical quality to the experts who developed said method. To facilitate improved method transfer, additional work was performed to reduce complex or error-introducing sample preparation steps and to reduce consumable consumption. The purpose of this research was to remove very specific method requirement that may introduce difficulty into analytical method replication by outside laboratories and analysts.

The aims of this additional research were to eliminate the requirement for venous blood-draw from the analysis of organic pollutants in blood, removing the requirement for consumable-heavy liquid nitrogen cryo-trapping in the analytical instrumentation, and the development of pre-calibrated SPE columns to remove the isotope-spiking step from the sample preparation procedure for environmental and biological samples.

A.2 Finger-stick Blood Draw – Undergraduate Teaching Laboratory

A.2.1 Introduction

The increasing personalization of medicine has resulted in the development of the term exposomics.[1] The field of exposomics combines clinical, environmental, and analytical chemistries to investigate how one's personal genetics defines his or her biochemical response to insult from environmental pollutants.[2] Recent studies have linked exposure to specific environmental agents with an increased risk of developing many non-communicable diseases.[3-9]

These agents, often called persistent organic pollutants (POPs), display common characteristics including bioaccumulation, environmental persistence, hydrophobicity, and inherent toxicity.[10] Emergent research and increasing clinical relevance have elevated the importance of understanding techniques used to accurately quantify these agents.

Blood and other biological fluids are clinically significant in assessing the environmental exposure of an individual.[11, 12] Quantitative clinical methods for POPs in whole blood and blood serum have been in use for decades.[13] However, existing methods requiring large volumes of blood are often not feasible for implementation in undergraduate teaching laboratories due to time required for venous collection, issues in obtaining internal review board (IRB) approval, cost, and biological hazards. A rapid, sensitive, and highly reproducible quantitative method was recently developed for the analysis of POPs in microliters of human blood.[14] It was hypothesized that the optimized method could be adapted for a teaching laboratory by the use of lancet finger-sticks in place of venous blood draws.

This method uses a solid-phase extraction technique called stir-bar sorptive extraction (SBSE), gas chromatography / mass spectrometry (GC/MS), and a direct quantification alternative to calibration curves called isotope dilution mass spectrometry (IDMS). Using these analytical techniques will allow undergraduate students to become familiar with modern analytical methods and, specifically, their application to highly complex biological matrices.

A.2.2 Pedagogical Considerations

Using their own whole blood, students will apply analytical techniques of POPs quantification and interpret the results to evaluate their individual profile of organic pollutant concentrations. Students are asked the question, "What is my individual profile of organic

pollutant concentrations?" and must defend their answer by describing the analytical process used and summarizing statistical results. Incorporation of biological sample handling, processing, and analysis provides students with a hands-on analytical experience that is often missing from undergraduate curricula. While reducing the potential for hazardous exposures, the use of each student's own blood also provides a contextualized chemistry education and motivates a personal investment in learning outcomes.

This laboratory can be used in courses related to or integrating clinical chemistry, environmental chemistry, and analytical instrumentation, with varying components of data analysis and statistics. This course is IRB-exempt for educations purposes. Anonymity of students, both in-class and in reporting results, was strictly maintained. This laboratory is intended for upper-class undergraduates who have completed traditional analytical courses. This experiment can be performed as two consecutive 3-hour laboratory sessions, with introductory and background information presented as a pre-lab in each session or as one separate 1-hour lecture. The compounds chosen for quantification represent ten commonly investigated environmental pollutants. The compound list may be altered in accordance to the primary literature source (see: Experimental Overview). This laboratory was conducted three separate times in a course module containing six students each. The laboratory contains a mixture of independent and group work, with each student expected to work individually on sample preparation, data analysis, statistics, and final lab reports. The method programming and instrumentation was performed as a group. This laboratory has several specific objectives. Following completion of this laboratory, students will have:

- Defined biological and chemical hazards
- Outlined the basic mechanisms and schematics of SBSE, GC/MS, and IDMS

- Applied standard analytical methods to collect and analyze blood samples
- Generated mean concentration, precision, limit of quantification, and confidence intervals for all compounds
- Defended their assessment of their personal profile of organic pollutants

A.2.3 Experimental Overview

Blood samples were obtained from students using lancet finger-stick devices and were then spiked with an isotopic quantification mixture.[15] The samples were extracted using SBSE and the concentrations of POPs were determined using GC-MS analysis and standard data mass spectrometer analysis software. A standard method of quantification called IDMS was used for direct, mathematical quantification.[16] While specific POPs were chosen for this laboratory, the source method allows for the use of any of a number of compounds that fall within a the given range of hydrophobicity and volatility (log $K_{O/W}$ = 2.3 - 7.5, log vapor pressure = -1 – 7 torr).[14]

Although students were familiar with basic instrumental and laboratory methods, a handout and pre-lab component served to contextualize the importance of clinical research (see: Supplementary Materials: Prelab). Students were instructed to review the referenced primary literature sources concerning high-sensitivity analytical methods and their application to clinical research. During laboratory work, the instructor or assistant was with students at all times.

A.2.4 Methods and Instrumentation

A.2.4.1 Reagents and Materials

For these experiments, 98% deuterium-labeled pendimethalin-d_5, acetochlor-d_{11}, metolachlor-d_6, benzo[a]pyrene-d_{12}, benzo[k]fluoranthene-d_{12}, dibenz[a,h]anthracene-d_{14}, and 99% deuterium-labeled benzyl butyl phthalate-d-$_4$ and diethylphthalate-d_4 were purchased from

C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Polydimethyl siloxane (PDMS) stir-bars (10 mm x 0.5 mm) were obtained from Gerstel (Mülheim a/d Ruhr, Germany). HPLC-grade acetonitrile (Sigma-Aldrich, St. Louis, MO) and ultrapure (18 Ω) water were used for reagent dilution, glassware cleaning, and stir-bar cleaning.

A.2.4.2 Standards Preparation

For each of the 10 isotopically labeled analytes, a stock solution was prepared in acetonitrile to approximately 500 μg/g. From these stock solutions, a calibration mixture was prepared in acetonitrile containing each isotopically labeled analyte at a final concentration of 10.0 μg/g.

A.2.4.3 Sampling

An accurately measured blood sample was obtained from each student. Each student used a new, sterile lancet to pierce his or her sterilized fingertip. A teaching assistant collected the blood with a pipette and transferred it to a 20 mL sample vial on a tared analytical balance. Up to three finger-sticks were required to obtain approximately 0.2 g of blood from each student. Three samples of 0.2 g were required. This process is demonstrated in figure A.1.

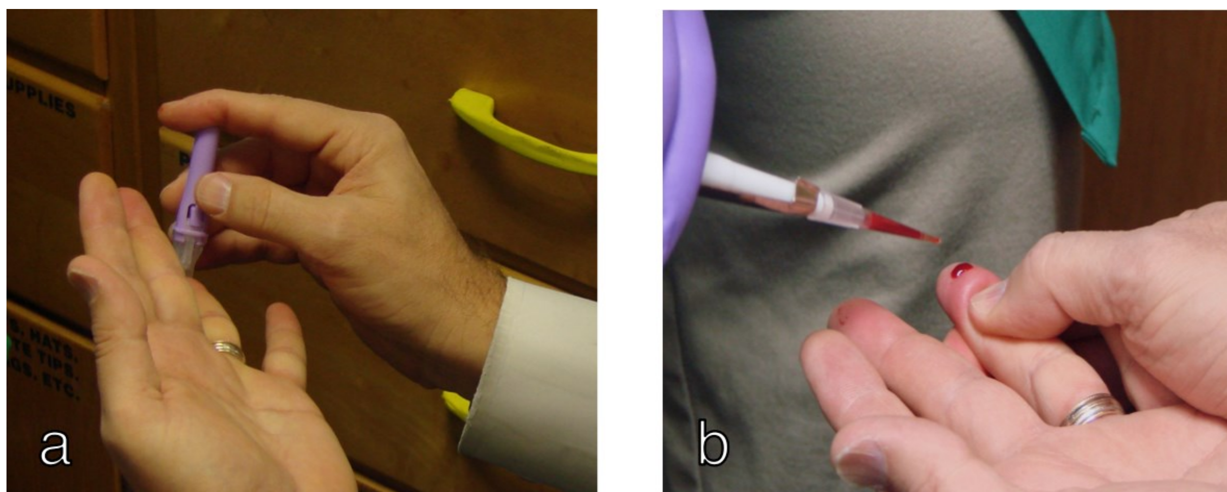

Figure A.1: The lancet finger-stick procedure showing a) the finger-stick using a sterile lancet on a sterilized finger pad and b) the collection of 0.2 g of blood by a teaching assistant using a pipette

A.2.4.4 Instrumentation

An accurately measured sample containing whole blood, isotopic calibration mixture, acetonitrile, and water were extracted using 10 mm x 0.5 mm PDMS stir-bars in 20 mL vials. Stir-bars were desorbed thermally in the thermal desorption unit (TDU) connected to the GC inlet at 300 °C under a splitless helium flow of 50 mL/min. Analytes were cryo-trapped using liquid nitrogen in the GC inlet using programmed-temperature vaporization mode. The analyte were released from the trap and separated on a 5%-phenyl PDMS column. Mass spectrometric analysis was conducted using electron ionization with a single quadrupole in select ion mode set to the quantitative ions found in literature for each compound. Using the provided qualitative software, peak areas were extracted for each natural and isotopically labeled analyte. Software developed in-house was used to quantitate each unknown analyte by IDMS. Detailed instrumental parameters can be found in Supplementary Material: Laboratory Instructions.

A.2.4.5 Pre-lab Preparation

Prior to the laboratory, each student was assigned a randomized identification number that was recorded only by the instructor and discarded upon the conclusion of the course. Students used this number to identify all samples, notebooks, and lab-reports in lieu of a name. The laboratory was stocked with three lancets per student, three alcohol swabs per student, antibacterial soap, and sterile drying cloths. Performing this laboratory in two days, the instructor must provide students with validation data on the specified analytes, as well as mass bias and $R_{i/n}$ values for each analyte.

A.2.4.6 Student Procedure

For the first 3-hour laboratory, students prepared each sample, spiking accurate amounts of isotopic calibrant, acetonitrile, water and one stir-bar into the vial containing the blood sample. While all samples extracted simultaneously for 60-minutes, students programmed the method parameters into the GC/MS according to the primary literature source.[14] Following extraction, students removed the stir bar from each sample, rinsed with water, and loaded into the auto-sampling cartridge of the GC/MS. The analyses were performed by automated sampling prior to the second day of experiments. During the second 3-hour laboratory, students extracted peak areas, given natural and isotopic *m/z* values and performed IDMS and statistical calculations.

This laboratory provides the option to expand to three experimental days. To expose students to validation and method development, the validation experiment, as well as mass bias and $R_{i/n}$ determinations, could be performed by students prior to the two days of experiments

described in this work. Details on these experiments can be found in Supplementary Materials: Laboratory Instructions.

A.2.5 Hazards and Administrative Considerations

The most significant hazards in this laboratory are biological in nature. All students were required to wear lab coats, gloves, and goggles at all times throughout the first. The teaching assistant, who was with the students in the laboratory at all times, obtained new, sterile gloves and pipette tips between sample collections from each student. Each student was required to sterilize the fingertip with an alcohol swab prior to sampling and to wash his or her hands with antibacterial soap after bleeding was stopped with a sterile swab. Basic first aid supplies were available in case of prolonged bleeding. All waste, including swabs, lancets, pipette tips, and sterile cloths, were deposited into a biological waste container. Upon completion of all sampling, the laboratory area, including balances and pipettes, was cleaned with a dilute bleach mixture. Students labeled each vial with their assigned number and were instructed to only handle their personal vials.

It was stressed to students that the experimental results were non-diagnostic and were neither intended nor capable of making medical inferences. Students with concerns on the medical implications of their results were instructed to direct their questions to a medical professional. To ensure compliance with the IRB of the institution, the students participating in this experiment remained anonymous by assignment of a computer-generated random number and were instructed to never place their name on vials, data files, laboratory notebooks, or lab reports. Any identifiable record linking the identification number of a student with a student name was destroyed following the conclusion of the course.

Students were presented with the option to not participate in the lancet finger-sticks and, instead, would be provided with a synthetically spiked, sterile sample. No student opted out of the laboratory. Prior to performing these experiments, instructors obtained written consent from each student, agreeing to participate in the experiment and allowing his or her data to be published as pooled results for future instructive purposes.

A.2.6 Results and Discussion

In this laboratory, students prepared, extracted, analyzed, and quantified samples of their own blood taken by lancet finger-sticks. Students performed IDMS and statistical analysis on the data extracted using the mass spectrometer qualitative software. Concentration data is discussed as approximate values to maintain anonymity and conform to the IRB. Each POP was detected in an average of 11 students, with a mean relative standard deviation of 13.3% between replicates (N=3). Class mean concentrations ranged from < ~0.002 μg/g blood to ~22.0 μg/g blood, with intra-analyte ranges up to four orders of magnitude. Mean limits of quantification were calculated by students, with a maximum sensitivity of 2 ng/g whole blood.

Students calculated personal mean concentrations, standard deviations, and 95% confidence intervals for each analyte, and performed a statistical outlier test of their choosing on each replicate. Calculations of limits of detection (calculated as 3-to-1 signal-to-noise) and quantification (calculated as 5-to-1 signal-to-noise) were performed by students for each analyte by extracting mean signal-to-noise ratios exported from the mass spectrometer qualitative software. Considering biological variance, the range and pattern of concentrations obtained by students fell within expected ranges observed in national studies. [14, 17] Pooled means of these

statistical values obtained from the analysis of samples from students, instructors, and teaching assistants can be found in table 1.

All reagents and instruments were validated by the instructor using the described experimental method. As the "true" value was unknown for each POP in the student's blood, accuracy was instead discussed by students in the context of the accuracy obtained in the validation procedure provided by the instructor.

Table A.1: Pooled class-wide means of statistical values calculated by students (all values in μg/g)

	Pooled Concentration[a]	%RSD	N[b]	Range[a]	LOD[c]	LOQ[d]
Pesticides						
Pendimethalin	<LOQ	N/A	0	N/A	0.03	0.05
Metolachlor	0.0100	10.9	13	0.00900 - 0.0700	0.00584	0.00974
Acetochlor	0.100	19.4	11	0.100- 19.0	0.09	0.15
Aromatic Hydrocarbons						
Dibenz[a,h])anthracene	0.0600	18.3	8	0.00700 - 0.0600	0.00444	0.00739
Benzo[a]pyrene	0.500	20.3	12	0.0100 - 2.00	0.0119	0.0198
Benzo[k]fluoranthene	2.00	8.1	12	0.00200 - 12.0	0.00123	0.00205
Naphthalate	<LOQ	N/A	0	N/A	0.00401	0.00668
Flurorene	<LOQ	N/A	0	N/A	0.00625	0.0104
Phthalates						
Benzobutyle phthalate	0.600	4.06	12	0.00300 - 3.00	0.00192	0.0032
Diethyl Phthalate	5.00	12.1	14	0.0100 - 22.0	0.0101	0.0169

a: Approximate mean concentration was used to maintain anonymity and conform to IRB
b: N refers to the number of students who demonstrated quantifiable amounts of the relevant compound
c: Limit of detection
d: Limit of quantification

Each student, identified with a unique, randomized numbers, demonstrated a unique pattern of exposure, with two students having no detectable quantities of any of the chosen POPs. Students prepared individual lab reports that addressed the stated laboratory objectives in a standard manuscript style (introduction, methods, results, discussion, conclusions). Students were expected to defend their analytical results based on calculated LOQ, confidence intervals, and validation data. Presented with the wide range of concentrations seen across the student

populations, students were provided with an opportunity to think critically on how personal genetics or environment may play a role in defining the patterns of concentrations observed in individuals exposed to relatively similar environments.

Robust studies on expected blood-concentrations for many POPs have not been conducted, but this laboratory demonstrated that finger-stick blood collection can provide adaquet sample-volume to achieve limits of quantification between 150 – 2 ng/g whole blood, a similar range to the source-method and national studies.[17] Finger-stick blood collections are a safer and lower-cost alternative to venous blood collections and allow undergraduates to experience hands-on learning with real biological samples.

A.2.7 Conclusions

This laboratory provided students with a hands-on opportunity to apply existing methodologies, interpret results, and evaluate their meaning using advanced analytical techniques. Finger-stick blood collection was used in lieu of venous blood draws to quantify clinically relevant organic pollutants, while minimizing cost, hazards, and sample handling. This laboratory included the use of advanced techniques of extraction, chromatography, mass spectrometry, and direct quantification in an experiment using real biological samples. Students reported that analysis of their own blood produced a high investment in learning outcome.

A.3 Removing Liquid Nitrogen Cryo-trap from Analytical Method

A.3.1 Introduction

A primary feature of the S-SBSE-IDMS described in Chapter 3 is the trapping of analytes, after volatilization in the TDU, with liquid nitrogen at -70 °C in the GC inlet.

Experiments have shown that volatile compounds adsorbed to a SBSE stir-bar tend to volatilize over the time of the TDU temperature ramp. This slow volatilization creates two problematic outcomes: irreproducibility of peak shape and size between replicates, and very broad chromatographic peaks. To address these problems, inert GC inlet liners are used as an area directly below the TDU schematically where gaseous compounds can be condensed using liquid nitrogen. This cryo-trap can then be ballistically heated to 300 °C to instantly volatilize all condensed compounds and introduce them onto the GC column to produce very narrow chromatographic peaks.

The cryo-trap process requires the use of liquid nitrogen to reach a temperature at which a high portion of gaseous analytes is condensed from the carrier gas flow coming from the TDU. In high-volume laboratories, cryo-trapping can use up to 180L of liquid nitrogen per week. This high rate of nitrogen consumption is both costly and logistically is difficult to coordinate the delivery of such large volumes of liquid gas to a laboratory on such a variable basis. It was the hypothesis of this work that the liquid nitrogen cryo-trap could be replaced by a thermoelectric, Peltier cooling device through the used of GC inlet liners packed with polymeric adsorptive materials. Inert GC inlet liners reply on one mechanism to trap gaseous molecules: condensation. However, adsorptive materials may add an additional trapping mechanism via a physical, non-covalent hydrophobic-hydrophobic interaction. The addition of this second trapping mechanism may allow gaseous molecule to be trapped at temperatures compatible with thermoelectric cooling: between -10 °C and 23 °C.

The purpose of this work was to investigate the potential for eliminating liquid nitrogen from the cryo-trapping step of SBSE - thermal desorption. Increasing the trapping temperature from the -70 °C typically used with inert inlet liners, such as quartz and glass wool, would allow

the use of a thermoelectric cooling system to reduce the associated maintenance and consumable costs. Cryo-trapping efficiency experiments were conducted between -70 and +30 °C.

A.3.2 Methods and Materials

Two types of adsorptive inlet liners included in these tests were supplied by Gerstel: Tenax TA and Carbopack B. The Tenax TA absorbent liners were used at varying cryo-trapping temperatures between -70 and +30 °C. Operational parameters and optimal trapping temperatures were determined and carry-over was assessed with method blanks between all replicate analyses, primarily based on the optimized method found in Chapter 3. The Carbopack B adsorptive liners were evaluated over these same temperature ranges.

For sample analysis, 8 uL of a 5 ug/mg mixture of representative EPA 625 compounds (excluding phenolic compounds, as they are incompatible with PDMS Twister bars) were directly spiked as a liquid into TDU desorption tubes and cryo-focused in the inlet at temperatures varying between -70 and +30 °C. A TDU primary desorption of 5 minutes at 300 °C and a CIS secondary desorption of 8 minutes at 300 °C were used for all measurements. An inlet split flow of 100 mL/minute (100:1 split) was used to sweep the trapped compounds from the inlet to the column. Method blanks were analyzed between every replicate to investigate potential carry-over. Carbopack B liners were then tested using identical parameters. Using the determined optimal temperature, the Tenax TA liner was used to test decreasing the split flow to reduce carrier gas consumption. A 10:1 split was compared to the standard 100:1 ratio.

A.3.3 Results[2]

A.3.3.1 Tenax TA Adsorptive Liner

Seven compounds were chosen for comparisons that represented low, medium, and high boiling point compounds, as well as a representative range of K_{ow} values. Benzene and toluene represented volatile compounds, trichlorobenzene and naphthalene represented semi-volatile compounds, and fluorene, hexachlorobenzene, and 4,4-DDD represented nonvolatile (at ambient conditions) compounds.

The adsorptive liner packed with Tenax TA was found to poorly retain compounds below -70 °C, the glass transition point of the polymer. A trapping temperature of -10 °C was found to provide optimum recovery from the spike for all selected compounds. Benzene recovery plateaued at -20 °C but provided indistinguishably high recovery at -10 °C and below. No carryover was observed in any of the method blanks. The limits of detection (calculated as 3:1 signal to noise ratio) of all selected compounds using the 100:1 split flow are plotted in figure A.2.

[2] All figures in Results section depict lower limit of detection- the smallest bars indicate the best performance.

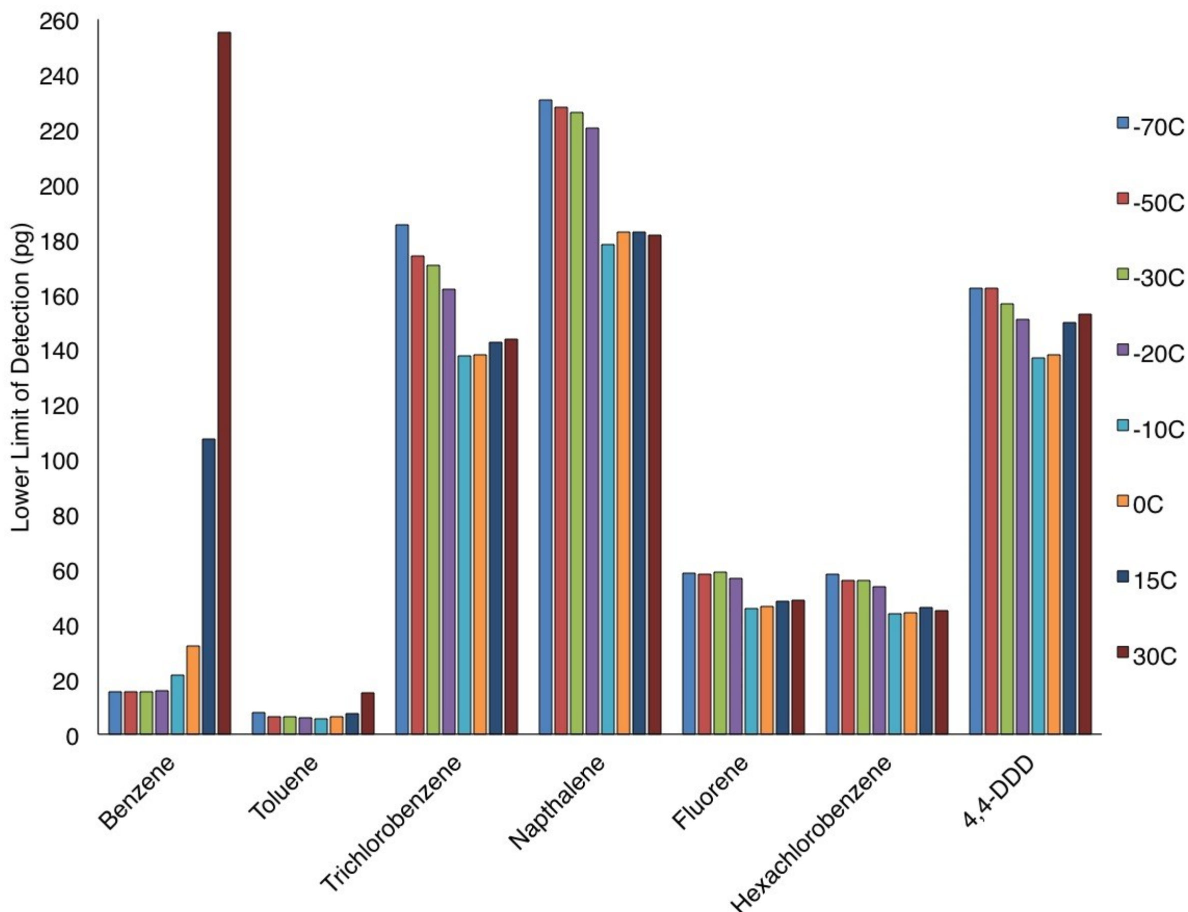

Figure A.2: Observed LOD values for selected analytes over a cryo-trapping range of -70 °C to +30 °C using Tenax TA inlet liners

A.3.3.2 Carbopack B Adsorption Liners

The adsorptive liners packed with Carbopack B were found to optimally retain the selected compounds at +15 °C. Analysis of benzene did not produce consistent peak areas, peak shape, or retention times above -50 °C. All blanks remained free of carry-over. The determined limits of detention (3:1 signal to noise) using the Carbopack B filled liners at 100:1 split ratio are noted in figure A.3.

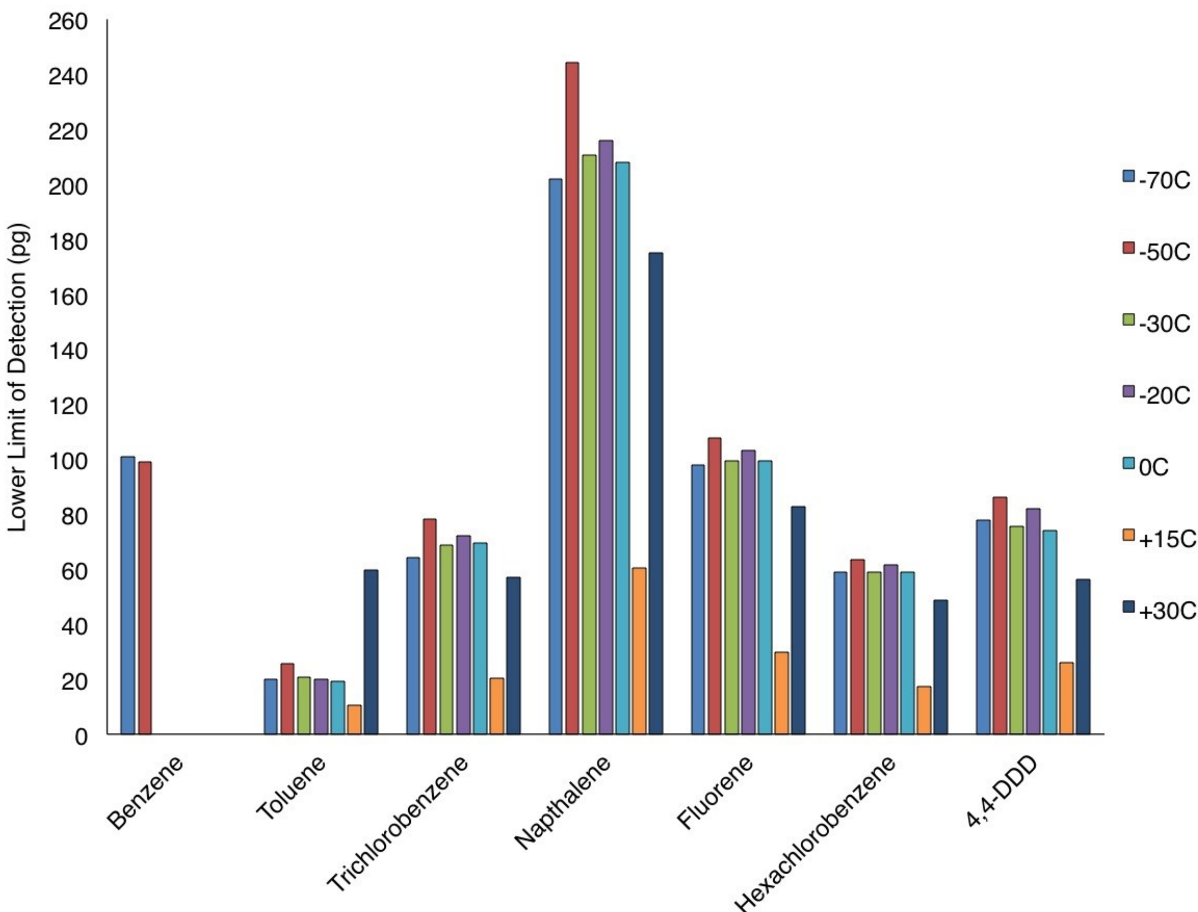

Figure A.3: Observed LOD values for selected analytes over a cryo-trapping range of -70 °C to +30 °C using Carbopack B inlet liners

A.3.3.3 Comparison Carbopack B and Tenax TA with Parameter Optimization

Comparison of spike recovery between Carbopack B and Tenax TA liners found that the Carbopack B retained a greater amount of medium and high boiling compounds while Tenax TA performed better for the very volatile analytes (benzene and toluene). These findings are demonstrated in figure A.4.

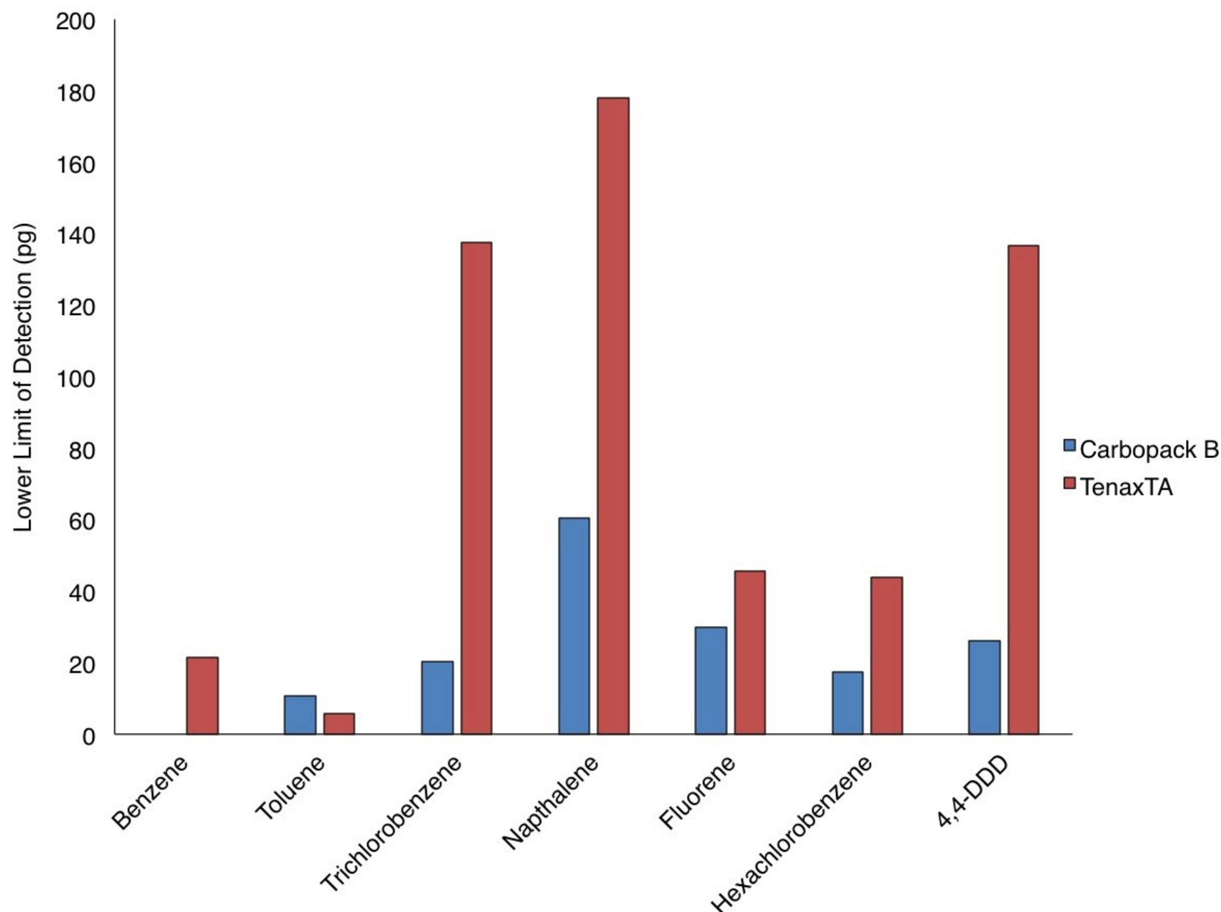

Figure A.4: Comparison of LOD values for selected analytes at the optimal trapping temperature for both Carbopack B (+15 °C) and Tenax TA (-10 °C) liners

Tenax TA provided the most stable capture and release of compounds from low to high boiling point and performed optimally at -10 °C, within Peltier cooling range. Using this cryo-trapping temperature, the inlet split ratio was successfully decreased down to 10 mL/minute (10:1) while continuing to prevent carry-over. The 10:1 split ratio also provided significantly better limits of detection for all compounds resulting in between 20% and 2700% improvement in sensitivity compared with the 100:1 split. These results indicate that optimal trapping across all studied analytes should use Tenax TA adsorptive liner trapping at -10 °C with a 10 mL/min (10:1) inlet flow. Summary of these findings can be found in figure A.5.

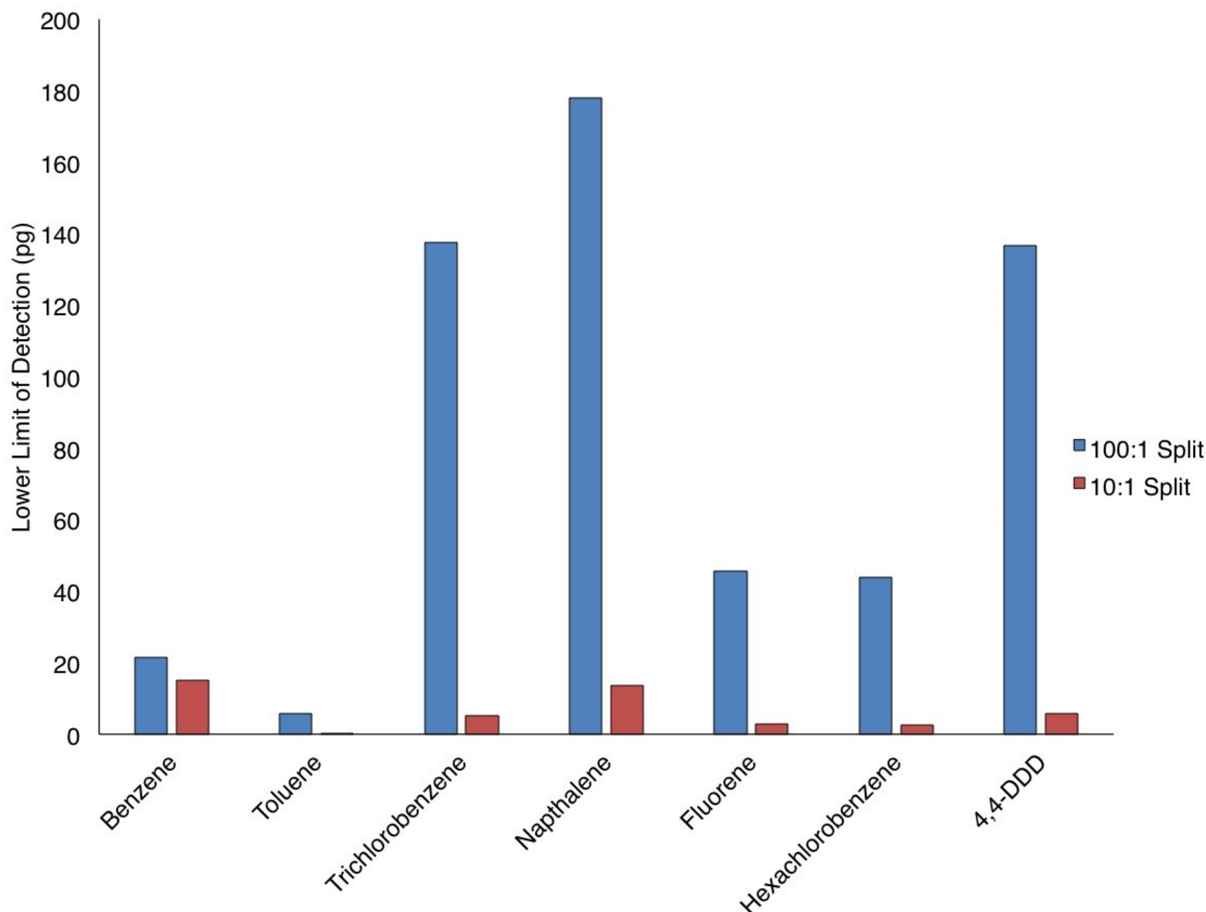

Figure A.5: A comparison between inlet split ratios using the optimal trapping temperature with Carbopack B liners

A.3.3.4 Conclusions

Experimental results have found that using a GC inlet liner packed with a polymeric adsorptive material can improve the efficiency an effectiveness of cryo-trapping such that higher temperatures may be used to produce optimal method sensitivity. A comparison was conducted between two common adsorptive-packed liners: Carbopack B and Tenax TA. It was found that, in comparison with results from Chapter 3, these liners increased the optimal cryo-trapping temperatures from -70 °C found with typical glass wool or quartz liners to -10 °C for Tenax TA and +15 °C for Carbopack B. The results for Carbopack B indicate that this polymer may allow analyte trapping via thermoelectric device in place of liquid nitrogen. The hypothesis that

adsorptive inlet liners may provide a way to eliminate liquid nitrogen cryo-trapping from the developed analytical method has been supported by this work.

A.3.3.5 Limitations and Future Work

The sensitivity of high boiling point compounds (>250 °C, fluorene and above) can theoretically be improved by increasing the temperature of the mass spectrometer source. The Agilent single quadrupole source has a default upper temperature limit of 250 °C - potentially causing condensation of high boiling point compounds from the gaseous phase.

Future work in this area should include an analysis incomplete desorption from the inlet liner. If the selected compounds are not recovered with 100% efficiency during secondary desorption, this could lead to more-frequent liner replacement. While these experiments successfully optimized the cryo-trap temperature to be within a Peltier cooling range, further tests must be done using a Peltier device to ensure that the process is field-ready.

A.4 Pre-calibrated Solid-phase Extraction Columns

A.4.1 Introduction

Implementation of high-quality mass spectrometry-based analytical methods has improved overall analytical accuracy, precision, and sensitivity attained in routine analyses.[18] Clinical and commercial laboratories have sought validated techniques to improve sensitivity, reproducibility, and accuracy through improved sample preparation, instrumental methodology, and quantitative procedures.[19, 20] To achieve higher analytical quality, many laboratories have adjusted analytical emphasis from common techniques, like assays, to higher-accuracy methods, like mass spectrometric identification and quantification.[21] However, without significant training,

many novel methods requiring complex sample preparation steps may not be replicated with the same quality by analysts or technicians in independent laboratories.[22] The methodological shift toward higher sensitivity and higher accuracy analytical methods[23, 24] has elevated the necessity for simplified sample preparation techniques reduce the influence of sample preparation steps on final analytical quality.

Often, quantitative accuracy is negatively impacted by errors introduced by the large number of highly precise sample preparation steps required in many methods and by inherent instrumental uncertainty.[25, 26] One technique used for reducing the influence of sample preparation and instrumental uncertainty on final quantitative quality is called isotope dilution mass spectrometry (IDMS).[27] IDMS is a quantification technique involving the spiking of accurate amounts of isotopically labeled analogs into an unknown sample. Using known isotopic abundances, concentration and mass of isotopically labeled spike, and mass of the unknown sample, the concentration of each analyte can be calculated mathematically without the use of calibration curves. IDMS can correct for many sources of error often associated with extraction, mass spectrometry, and quantification. These common sources include imprecise sample preparation, poor extraction reproducibility, sample loss, low analyte recovery, instrumental drift, matrix effects, and physical or chemical interferences. Much of the error introduced in sample preparation to change the concentration of natural analyte in a sample will affect the isotopic analog identically and be corrected in the final IDMS equation.[27, 28] By reducing the influence of common error-introducing analytical steps on final quantitative quality, IDMS is capable of transferring high accuracy methods between laboratories and analysts with minimal additional training.[29] However, in-laboratory spiking of the isotopically labeled analogs into the

unknown samples remains a potential source of analytical error when transferring developed methods using IDMS.

Commonly, laboratories analyzing environmental and forensic samples utilize gas chromatography / mass spectrometry (GC/MS), liquid chromatography / mass spectrometry (LC/MS), or the enzyme-linked immunosorbent assay (ELISA).[30-32] However, with increased availability, many laboratories have begun adopting sensitive and high-resolution instrumentation like time-of-flight mass spectrometers (TOF-MS) to eliminate the requirements of derivatization and chromatography.[33, 34] With the advent of highly sensitive analytical methods, sample cleanup has become an important part of sample preparation to reduce harmful biological material and to pre-concentrate the analytes of interest.

Solid-phase extraction (SPE) is a sample cleanup and pre-concentration procedure to isolate analytes of interest from potentially interfering or harmful biological compounds using primarily hydrophobic interactions and cationic or anionic exchange.[35] SPE is often used on aqueous and biological samples to selectively extract and pre-concentrate analytes prior to analysis by LC/MS or electrospray ionization (ESI) TOF-MS. It was hypothesized in the research presented here that modified SPE columns could be pre-calibrated by, prior to analysis of an unknown sample, the loading of accurately known concentration of isotopically labeled analogs by highly skilled analysts. Pre-calibrated columns may be useful in transferring analytical quality between and among laboratories by removing the spiking of isotopically labeled analogs from the in-laboratory sample preparation, potentially further reducing the influence of sample preparation on final quantitative quality. This broad analytical process is diagrammed in figure A.3.

This research focused on developing a SPE pre-loading method for future implementation in improving the transfer of high accuracy and precision analytical methods between and among laboratories. For optimization experiments, the environmentally relevant pesticide glyphosate was chosen for its high usage rates, analytical difficulty, and inclusion in national drinking water regulations. Glyphosate is a polar organophosphate pesticide extensively used in the U.S. for vegetation control. The hydrophilicity and ionic character of this molecule make quantification in aqueous solution difficult. The U.S. Environmental Protection Agency (USEPA) has regulated a maximum contaminant limit of glyphosate in drinking water of 0.7 μg/mL.[36] Following development, optimization, and validation for the pesticide glyphosate, the potential forensic applications were explored by analysis of seven drugs of abuse in synthetic urine. For both glyphosate and the drugs of abuse, experiments were performed to assess quantitative stability over time to simulate future experiments in which pre-loaded SPE columns will be shipped to off-site laboratories for analysis.

A.4.2 Experimental

A.4.2.1 Chemicals and Standards

Glyphosate (99% pure) and glyphosate-2-^{13}C (99% pure, 99% enriched) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Drinking water samples were supplied by Pittsburgh Municipal Water (Pittsburgh, PA, USA). Heroin, 6-AM, morphine, cocaine, methadone, and fentanyl analytical standards at certified concentrations of 1.0 mg/mL and codeine analytical standard at a certified concentration of 100 μg/mL were purchased from Cerilliant (Round Rock, TX, USA). The deuterium-enriched analogs: heroin-D_9, morphine-D_3, cocaine-D_3, codeine-D_3, methadone-D_3, and fentanyl-D_5 at certified concentrations of 100 μg/mL and 6-AM-D_3 at a

certified concentration of 1.0 mg/mL were purchased from Cerilliant. Synthetic urine, HPLC grade methanol, HPLC grade water, Hyclone phosphate buffered saline (PBS), HPLC grade 2-propanol, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetate buffer was prepared using sodium acetate and acetic acid purchased from Fisher Scientific. Each naturally occurring analyte was prepared in separate stock solutions at 10 µg/mL for the drugs of abuse and 60 µg/mL for glyphosate in HPLC grade water. For glyphosate, two solutions were prepared by mass in drinking water: one spiked with 6 µg/mL of glyphosate and one spiked with 6 µg/mL of glyphosate-2-^{13}C. Two solutions were prepared by mass in synthetic urine for the drugs: one containing all unlabeled drugs at 40 ng/mL and one containing all isotopically labeled analogs at 40 ng/mL.

A.4.2.2 Solid-Phase Extraction

For glyphosate experiments, Strata-SAX SPE columns (500 mg bed mass, 6 mL volume) were purchased from Phenomenex (Torrance, CA, USA) for the analysis of glyphosate in drinking water. Columns were conditioned per manufacture recommendations with 4.0 mL HPLC grade methanol and 4.0 mL HPLC grade water. Then, 4.0 mL of drinking water sample was loaded onto the column and washed with 4.0 mL of HPLC grade methanol. Elution was performed with 16 mL of a 1:1 solution of acetonitrile and methanol acidified at 6% with formic acid. All SPE analyses were performed with a negative pressure vacuum chamber at 1 mL/min. For IDMS quantification, a solution of glyphosate-2-^{13}C in HPLC grade water was loaded onto the column after conditioning but before the spiked drinking water sample was loaded.

For the drugs of abuse, CSDAU303 (UCT, Bristol, PA) SPE were used for extraction of drugs from synthetic urine. Columns were conditioned with 2.0 mL of HPLC methanol, 2.0 mL

HPLC water, and 2.0 mL PBS. Extraction was performed on 4.0 mL synthetic urine spiked with unlabeled analytes, followed by a wash of 4.0 mL water, 3.0 mL acetate buffer, and 3.0 mL of methanol. For IDMS experiments, a 4.0 mL aliquot of the isotopically enriched working solution (phosphate-buffered to pH 6) in synthetic urine was loaded onto the column next. After a two minute on-column drying period, all extracted compounds were eluted from the column using 11.0 mL ethyl acetate:2-propanol:ammonium hydroxide (84:12:1) solution.

A.4.2.3 ESI-TOF-MS

A Bruker Daltonics microTOF (Billerica, MA, USA) mass spectrometer with an orthogonal ESI source was experimentally optimized for the analysis of all analytes of interest and their deuterium-enriched analogues. Samples were infused using the ESI source at a flow rate of 240 μL/hour with a Cole Palmer 74900-00 syringe pump (Vernon Hills, IL). The experimentally optimized method parameters can be found in the Results section. Quantitative *m/z* ions were experimentally found for each labeled and unlabeled analyte. Quantitative ions for each natural and isotopic compound can be found in table A.2.

Table A.2: Quantitative ions for each studied analyte and the corresponding exact and measured masses

Analyte	Quantitative Ion	Exact Mass (*m/z*)	Measured Mass (*m/z*)
Glyphosate	$C_3H_7NO_5P^-$	168.00618	168.00673
Morphine	$C_{17}H_{20}NO_3^+$	286.14432	286.15051
Codeine	$C_{18}H_{22}NO_3^+$	300.15997	300.16225
Cocaine	$C_{17}H_{22}NO_4^+$	304.15488	304.15816
Methadone	$C_{21}H_{28}NO^+$	310.21709	310.22002
6-AM	$C_{19}H_{22}NO_4^+$	328.15488	328.15521
Fentanyl	$C_{22}H_{29}N_2O^+$	337.22799	337.2277
Heroin	$C_{21}H_{24}NO_5^+$	370.16545	370.16887
Glyphosate-2-13C	$C_2{}^{13}C_1H_7NO_5P^-$	169.01485	169.01455
Morphine-D3	$C_{17}H_{17}D_3NO_3^+$	289.17031	289.1708
Codein-D3	$C_{18}H_{19}D_3NO_3^+$	303.18596	303.18769
Cocaine-D3	$C_{17}H_{19}D_3NO_4^+$	307.18088	307.18249
Methadone-D3	$C_{21}H_{25}D_3NO^+$	313.24308	313.24389
6-AM-D3	$C_{19}H_{19}D_3NO_4^+$	331.18088	331.1833
Fentanyl-D5	$C_{22}H_{24}D_5N_2O^+$	342.27131	342.27417
Heroin-D9	$C_{21}H_{15}D_9NO_5^+$	379.24343	379.24523

A.4.2.4 SPE Pre-loading

SPE column pre-loading followed the same conditioning and washing procedures for the separate columns listed in the Solid-Phase Extraction section. For pre-loading, matrix-matched solutions containing isotopic analogs were extracted and allowed to air-dry for a determined period prior to extraction of solutions spiked with certified concentrations of unlabeled analytes.

Optimization of pre-loading procedure assessed three methods of storing columns: allowing columns to air-dry (AD) in an upright position, rinsing the columns with 2 mL of HPLC grade water (WR) and storing wet with Parafilm sealing both ends of the column, and rinsing the columns with 2 mL of HPLC grade methanol (MR) and sealing with Parafilm. Two sample extraction modifications were assessed: decreasing the volume (DV) of the isotopically labeled solution from 4 mL to 200 µL, and placing an individual frit (IF) approximately 2 cm above the column packing. Assessment of these techniques followed a reasonable order. The storage methods were testing first and comparisons were generated between all three. The optimal method that provided the greatest long-term quantitative stability was used for all future

analyses. Pre-loaded columns were cold-stored at 15 °C for one week, two weeks, and four weeks prior to analysis of a solution of drinking water spiked with certified concentrations of glyphosate. Five replicates we performed for each technique at each time interval.

A.4.2.5 Method Validations

For validation experiments, solutions were prepared by spiking certified standards for each analyte being studied into either drinking water or synthetic urine matrix by mass. The appropriate sample preparation was applied (either SPE using the above method or SPE pre-loading using the above method) and the eluate was analyzed using the above method for ESI-TOF-MS. Quantification was performed by IDMS. Replicates were performed for all validation experiments (n=20 for glyphosate by SPE and SPE pre-loading, n=5 for drugs by SPE and SPE pre-loading). Experimentally obtained values were compared with calculated values at the 95% confidence level. For assessment of accuracy, an adequate percent error to be achieved by the method being validated was set at ±10%.

A.4.3 Results

A method for the quantification of glyphosate using ESI-TOF-MS, traditional SPE, and IDMS quantification were validated by adapting and optimizing existing methods.[27, 37] Validation results using IDMS quantification were compared with calibration data. Various techniques were assessed for increasing the quantitative stability over time of columns pre-loaded with glyphosate-2-^{13}C. The optimal pre-loading technique was then validated in drinking water samples spiked with unlabeled glyphosate analyzed with the pre-loaded SPE columns. Robustness of the pre-loading method was then assessed for potential forensic application by

adapting and optimizing the instrumental method for seven drugs of abuse in synthetic urine. Quantitative stability over time for the SPE pre-loading method was assessed for both environmental and forensic applications.

A.4.3.1 Optimization of SPE and ESI-TOF-MS

Strata-SAX (Phenominex) columns were used in the experimental optimization of SPE elution volume and pH. It was found that the optimum efficiency of glyphosate elution occurred at pH 6.0. Therefore, an eluting solvent consisting of 1:1 acetonitrile/methanol acidified to 6% formic acid was used. Analysis of eluent fractions determined 16 mL was required to elute the 4 mL of 6 µg/mL solution loaded onto the SPE column.

Instrumental parameters were optimized for signal intensity using negative ionization ESI-TOF-MS. Experimentally optimized instrumental parameters for drinking water samples spiked with glyphosate can be found in table 1.

Table A.3: Experimentally optimized mass spectrometry parameters for the quantification of glyphosate.

Parameter	**Value**	**Parameter**	**Value**
Ionization Mode	Negative	*Dry Temperature*	200 °C
Scan Range	50 - 1000 *m/z*	*Capillary Exit*	-100 V
Endplate Offset	-500 V	*Skimmer 1*	-40.0 V
Capillary Voltage	+3750 V	*Hexapole 1*	-23.0 V
Nebulizer	0.5 Bar	*Hexapole RF*	65 Vpp
Dry Gas	4.0 L/min	*Skimmer 2*	-22.0 V

Accurate quantification was attained using isotopic distribution patterns, expected *m/z* shift between glyphosate and glyphosate-2-^{13}C, and mass accurate resolution of glyphosate and glyphosate-2-^{13}C.[38] For unlabeled glyphosate, a mean mass accuracy of 3.2 ppm was obtained with mean deviation of 4.3 ppm. For glyphosate-2-^{13}C, a mean mass accuracy was obtained of

1.7 ppm with a mean deviation of 4.1 ppm. At 168 *m/z*, a resolving power of 18,000 m/Δm was obtained. Mass bias was calculated to determine the differential instrument response between the natural and isotopically labeled forms of glyphosate. A mass bias factor of 1.0006 was determined for the IDMS quantification procedure for glyphosate.[39] Optimized instrumental parameters produced the resolved spectra seen in figure A.6.

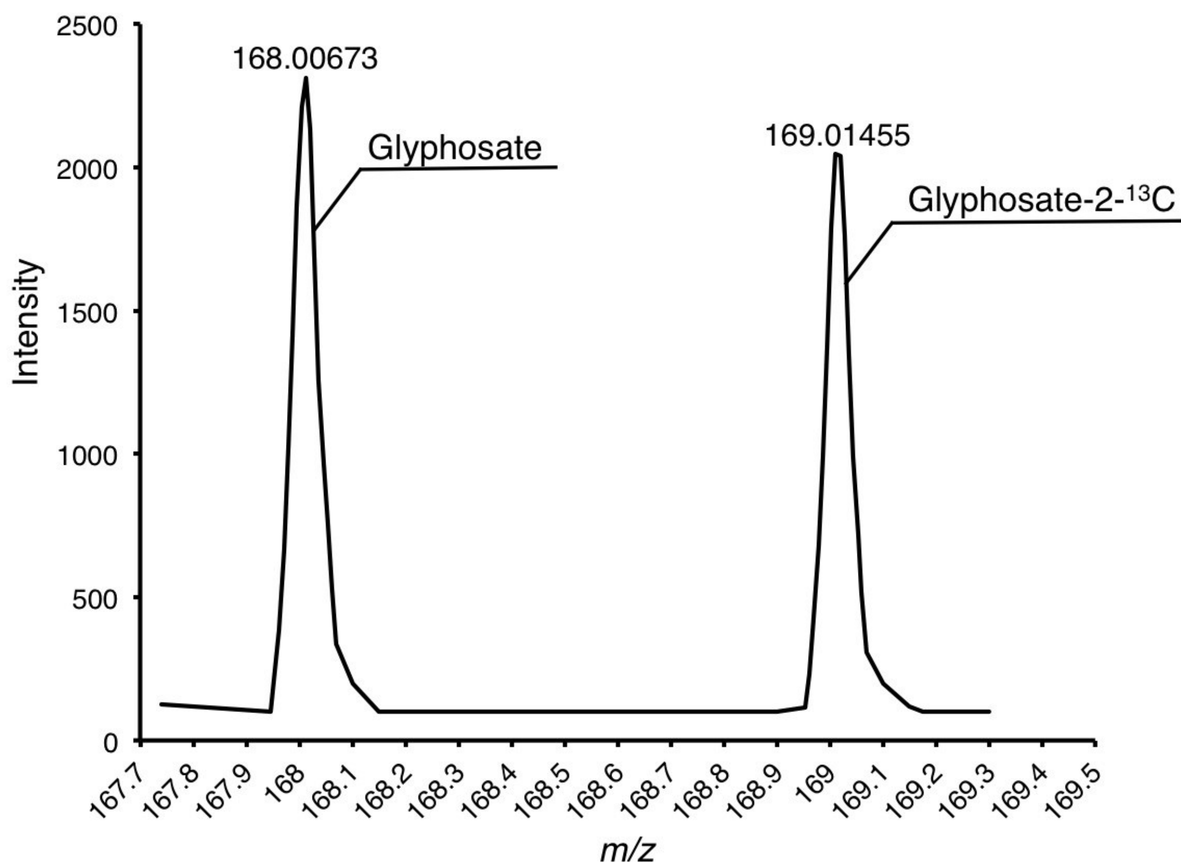

Figure A.6: Averaged mass spectra (n=7) obtained from the analysis of drinking water spiked with 50 μg/mL of glyphosate and 50 μg/mL of glyphosate-2-^{13}C showing baseline resolution and mass accuracy

While high-resolution mass spectrometry (HR-MS) was used for mass accurate compound identification in this research on simple samples containing known compounds, it has been reported elsewhere that mass accuracy, even below 1 ppm experimental mass error, is not

sufficient to accurately identify unknown analytes in samples.[40] This research optimized an instrumental method for the quantification of glyphosate in known samples only as a means of assessing the developed pre-loading technique and the mass accuracy of 3 ppm was therefore deemed adequate. This optimized instrumental method would not be suitable for absolute identification of samples containing unknown compounds.

A.4.3.2 Validation of SPE and ESI-TOF-MS

Instrument limit of quantification (LOQ) of ESI-TOF-MS for glyphosate in drinking water was found to be 0.312 μg/mL. Method LOQ for ESI-TOF-MS using traditional SPE and IDMS quantification for glyphosate in drinking water was found to be 0.401±0.01 μg/mL. Using IDMS, the experimentally determined LOQ was found to be equal to the experimentally determined limit of detection (LOD), confirming previous studies on molecular IDMS quantification.[29] This LOQ sufficiently exceeded the National Primary Drinking Water Regulations from the USEPA, which specified a maximum glyphosate contaminant level of 0.700 μg/mL.

Drinking water spiked with glyphosate standard at a certified concentration of 6.00 μg/mL was analyzed using the optimized ESI-TOF-MS parameters and the traditional SPE, quantified using IDMS (n=20). The optimized method produced an experimental concentration of 5.95±0.08 μg/mL, representing an overall accuracy with 0.83% error and a precision of 1.3% RSD. These results indicate that a valid, optimized instrumental method was adapted from previously published sources to provide precise and mass-accurate quantification to be applied to the assessment of SPE pre-loading techniques.

A.4.3.3 Comparison of Quantitative Methodologies

Samples spiked with both labeled and unlabeled analytes at concentrations approaching the LOQ were used to create a calibration curve. Figure A.7 shows calibration data approaching the determined LOQ as well as the same data treated with IDMS quantification from the same samples, showing percent error from certified concentration, with 95% confidence intervals, at various concentrations points (n=5 at each point). Quantitative methodologies were compared using the validated ESI-TOF-MS method with traditional SPE. Drinking water samples spiked with certified concentrations of glyphosate were quantified using IDMS with a mean accuracy of 4.22%±5.81% error over the concentration range 0.600 – 6.25 μg/mL, losing quantitative accuracy below 0.401 μg/mL. Calibration curve quantification lost quantitative accuracy at the 95% confidence level at 3.25 μg/mL, exhibited 59.4%±43.0% error the studied concentration range, but remained detectable to a concentration of 0.401 μg/mL. IDMS and calibration curve quantification demonstrated identical limits of detection, but IDMS produced a significantly lower LOQ.

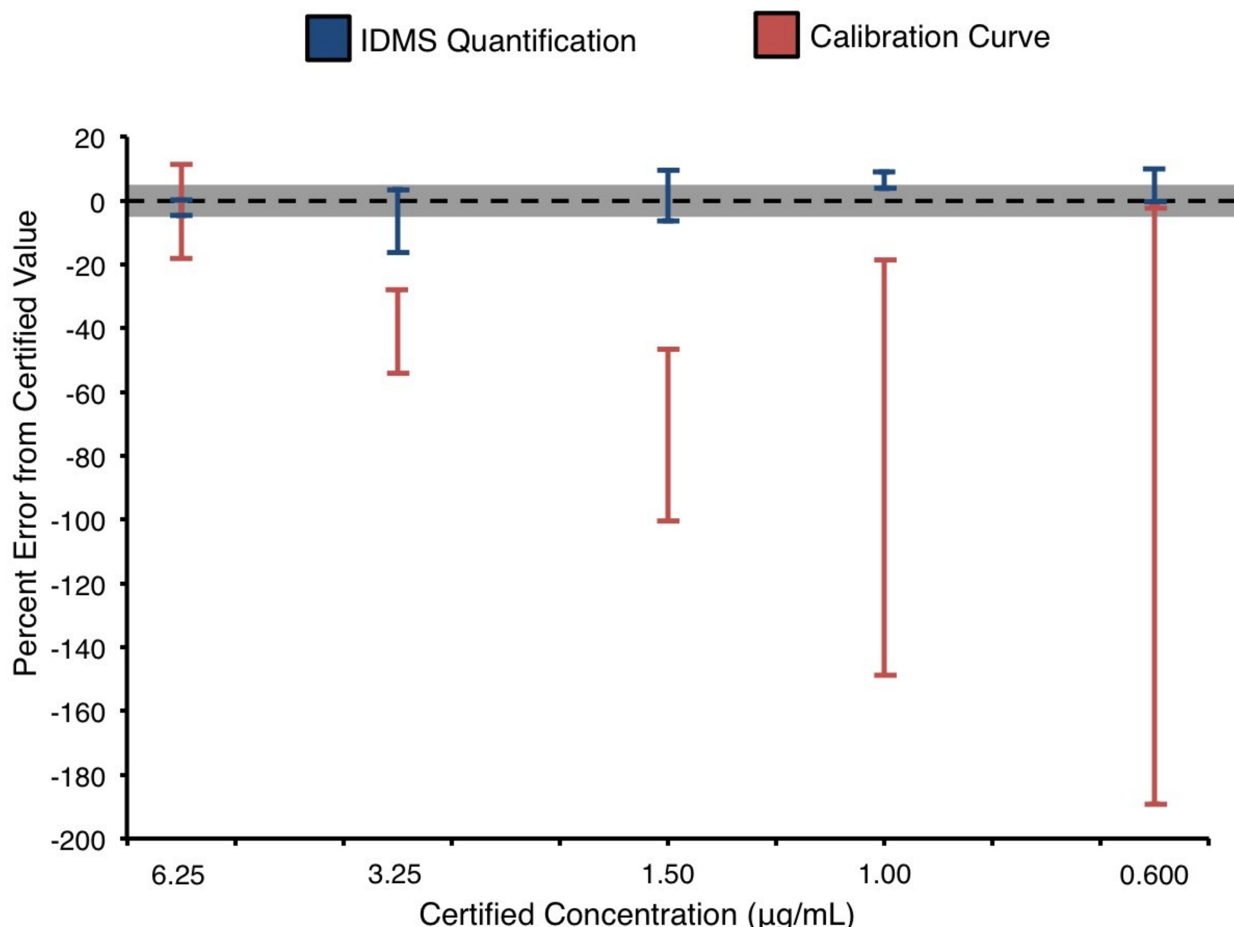

Figure A.7: A comparison of percent error of glyphosate quantification obtained in drinking water at various concentrations using IDMS and calibration curve quantifications. Dashed black line indicates certified value and shaded area represents ±5% certified error.

A.4.3.4 Optimization of Pre-loading Method

Samples containing glyphosate-2-^{13}C in drinking water were loaded onto the SPE columns using the manufacturer recommended procedure. These pre-loaded columns lost adequate accuracy when used for quantification of spiked drinking water samples after 1 day of storage. Several optimization procedures were then undertaken to increase on-column stability. The techniques that meaningfully impacted percent error are summarized in figure A.8, showing absolute value of percent error using each specified method with 95% confidence intervals.

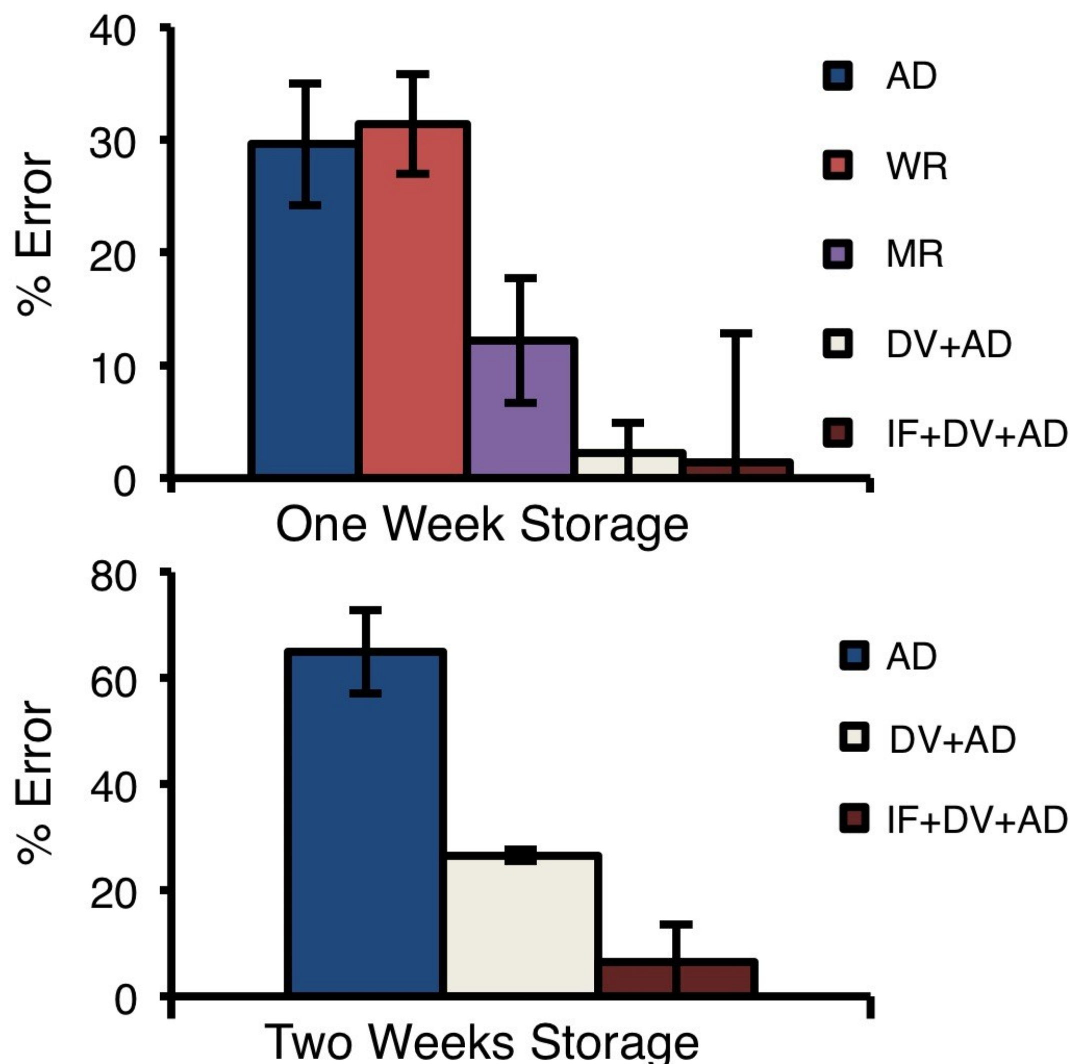

Figure A.8: The effects on absolute value of percent error of multiple optimization techniques after (top) one week of storage and (bottom) two weeks of storage, with 95% confidence intervals shown

Three techniques of storing SPE columns loaded with glyphosate-2-^{13}C: air-drying columns (AD), storing with 2 mL water-rinse (WR), and storing with 2 mL methanol-rinse (MR) were assessed for quantitative stability over time. Two modifications of applying the pre-loading spikes were assessed: decreasing volume from 4 mL to 200 µL of spiking solution (DV), and spiking the solution onto an individual frit (IF) above the column packing. Combinations of storage conditions and pre-loading techniques were assessed in the optimization experiments.

The optimization experiments were performed using 5 μg/mL of pre-loaded glyphosate-2-^{13}C and 5 μg/mL of glyphosate (n=5 for each technique).

At one week of storage, AD and WR showed no statistical difference in percent error, but MR exhibited statistically decreased percent error from both AD and WR ($p<0.05$). However, both WR and MR produced disruption of the SPE column packing material, which lead to irreversible binding of glyphosate-2-^{13}C. Irreversible binding of the pre-loaded isotope was suspected in WR and MR by the production of large positive bias after storage for two weeks. Irreversible binding was confirmed by the observation of large negative bias by pre-loading unlabeled analytes and quantifying a solution of isotopically labeled analytes after two weeks of storage. Therefore, WR and MR were eliminated from the optimization experiments. The modified pre-loading procedure of DV+AD significantly reduced percent error compared with AD, WR, and MR (all $p<0.05$). The modified pre-loading procedure of IF+DV+AD produced significantly decreased percent error compared with AD and WR (all $p<0.05$).

After two weeks of storage, all optimization techniques increased in percent error compared with the same technique after one week. DV+AD exhibited significantly lower percent error compared with AD after two weeks of storage ($p<0.05$), but did not demonstrate adequate quantitative accuracy (26.6%±1.12% error). IF+DV+AD exhibited further significant decrease in percent error compared with DV+AD ($p<0.05$) and maintained adequate quantitative accuracy (6.41%±7.14% error).

When extended to four weeks of storage, no technique or combination of techniques yielded adequate quantitative accuracy, with IF+DV+AD exhibiting 47.2%±1.35% error. Results for IF+DV+AD over four weeks can be found in figure A.9. Significant loss of stability was observed between two and four weeks for pre-loaded columns. It was determined that the

combination IF+DV+AD would be used in the pre-loading method to maintain high-quality quantification up to two weeks after pre-loading. Future research will work to improve the stability of on-column analyte stability during storage.

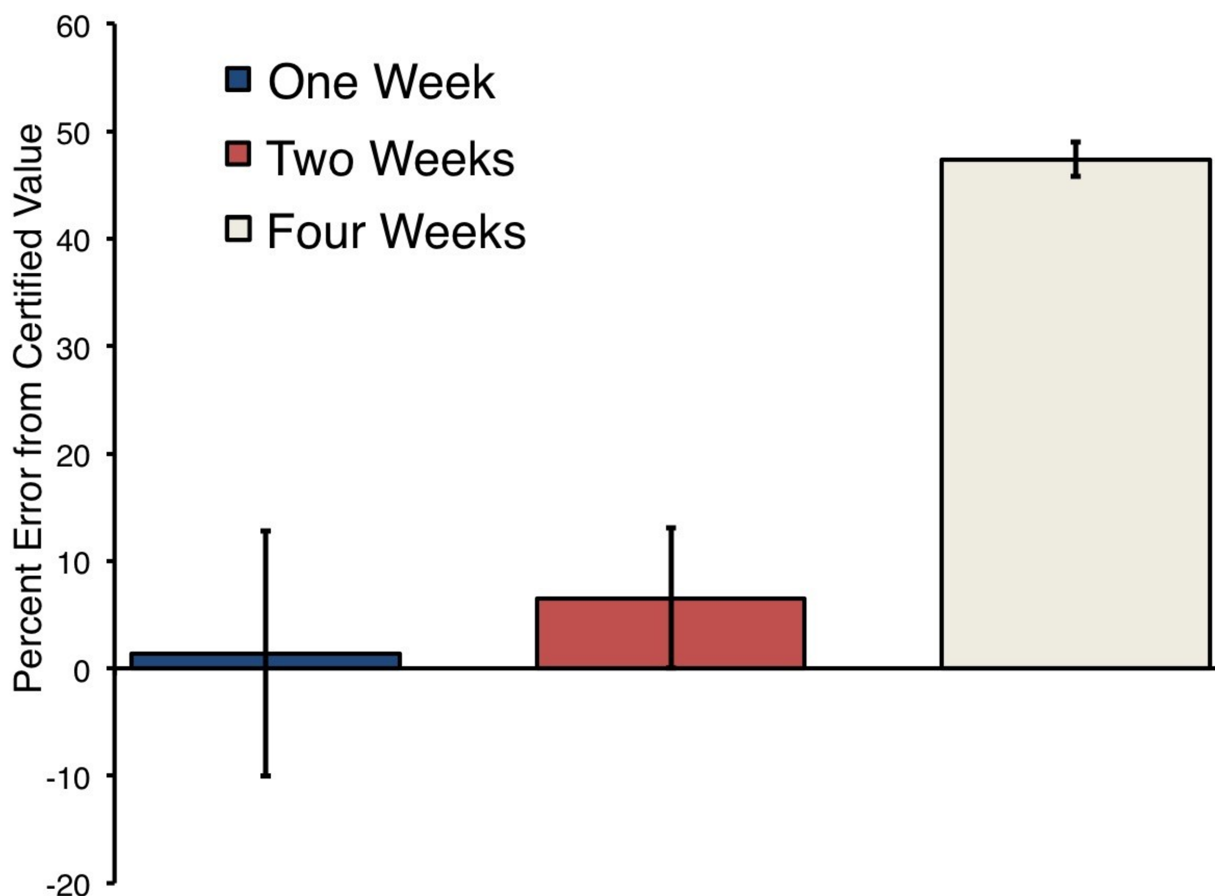

Figure A.9: Quantitative stability over time of SPE columns pre-loaded with isotopically labeled glyphosate. Percent error and 95% confidence errors are shown.

A.4.3.5 Validation of Pre-loading Technique

Using the validated instrumental parameters from the optimized ESI-TOF-MS method and the IF+DV+AD technique optimized for SPE pre-loading, a validation experiment was performed on drinking water spiked with 6 µg/mL of glyphosate (n=20). Columns were pre-loaded with isotopically labeled glyphosate at a concentration of 6 µg/mL 30 minutes prior to analysis of the spiked drinking water to allow the columns time to air-dry. Drinking water

samples analyzed 30-minutes after pre-loading columns with isotopes produced a final concentration of 5.93±0.052 μg/mL, representing a 1.25% error and a 0.87% RSD. A method LOQ of 0.401 μg/mL was obtained for the pre-loaded columns, identical to the LOQ obtained for ESI-TOF-MS using traditional SPE. These results indicate that the IF+DV+AD pre-loading technique produced valid quantitative results that did not differ significantly from either the certified value or quantitative results from the ESI-TOF-MS method using traditional SPE at the 95% confidence level.

A.4.3.6 Potential Application to Drugs of Abuse in Synthetic Urine

The optimized and validated ESI-TOF-MS analytical method was optimized for the quantification in synthetic urine of seven common opioids and alkaloids used as adulterants. Experimentally optimized instrumental parameters can be found in table A.4. Analysis of these opioid and alkaloid compounds required changing the SPE column from the anion-exchange column used for glyphosate to a Clean Screen DAU (UCT) mixed-mode hydrophobic and cation-exchange column.

Table A.4: Experimentally optimized mass spectrometry parameters for the quantification of opioid and alkaloid drugs of abuse.

Parameter	**Value**	**Parameter**	**Value**
Ionization Mode	Positive	*Dry Temperature*	200 °C
Scan Range	240-400 *m/z*	*Capillary Exit*	135 V
Endplate Offset	-500 V	*Skimmer 1*	40.0 V
Capillary Voltage	-4500 V	*Hexapole 1*	23.0 V
Nebulizer	0.4 Bar	*Hexapole RF*	250 Vpp
Dry Gas	4.0 L/min	*Skimmer 2*	24.0 V

The optimized instrumental parameters produced valid quantification for all seven drugs spiked in synthetic urine at 40 ng/mL following traditional SPE extraction. Validation results for

ESI-TOF-MS using traditional SPE and IDMS can be found in table A.5 comparing certified concentrations with experimentally determined concentrations with 95% confidence intervals. All drugs of abuse were quantified with <10% quantitative error and <20% RSD. Accuracy ranged from 0.689% to 9.33% error for methadone and cocaine, respectively. Precision ranged from 0.973% to 11.6% RSD for fentanyl and morphine, respectively. Mean quantitative accuracy for samples analyzed using ESI-TOF-MS with traditional SPE exhibited mean accuracy of 4.16%±3.07% error and mean precisions of 5.93%±3.03% RSD. Experimentally determined concentrations did not differ significantly from certified concentrations at the 95% confidence level.

Table A.5: Validation values for opioids and alkaloids using ESI-TOF-MS, traditional SPE, and IDMS quantification for drugs of abuse in synthetic urine, showing 95% confidence

SPE-ESI-TOF-MS (n=5)	Calculated Value (ng/mL)	Experimental Value (ng/mL)	|%Error|	%RSD	LOD (ng/mL)	LOQ (ng/mL)
Morphine	40±2.00	42.2±6.65	5.57	11.6	0.446	0.800
Codeine	40±2.00	42.3±4.44	5.86	7.73	0.202	0.800
Cocaine	40±2.00	43.7±2.18	9.33	3.69	0.773	0.800
Methadone	40±2.00	39.7±2.32	0.689	4.32	0.128	0.800
6-AM	40±2.00	40.3±0.531	0.797	6.12	0.0502	0.800
Fentanyl	40±2.00	42.4±4.10	0.710	0.973	0.234	0.800
Heroin	40±2.00	42.2±6.65	6.18	7.13	0.238	0.800

The method LOQ determined for the drugs of abuse using the optimized ESI-TOF-MS with traditional SPE and IDMS of 0.780 ng/mL for all analytes. As with the work with glyphosate, quantification with IDMS produced significantly higher accuracy and precision approaching the LOQ. In figure A.10, IDMS and typical calibration curve quantitation are compared in the same samples using the validated ESI-TOF-MS method and traditional SPE, showing mean percent difference from certified concentrations of all analytes included in this study at various concentration points, with 95% confidence ranges (n=5). IDMS exhibited a

mean percent error across all concentration points for all analytes of 5.66%±10.9%. Calibration curve lost quantitative accuracy below 3.13 ng/mL, exhibiting a mean percent error for the concentrations range 25.0 ng/mL through 3.13 ng/mL of 11.5%±27.8%.

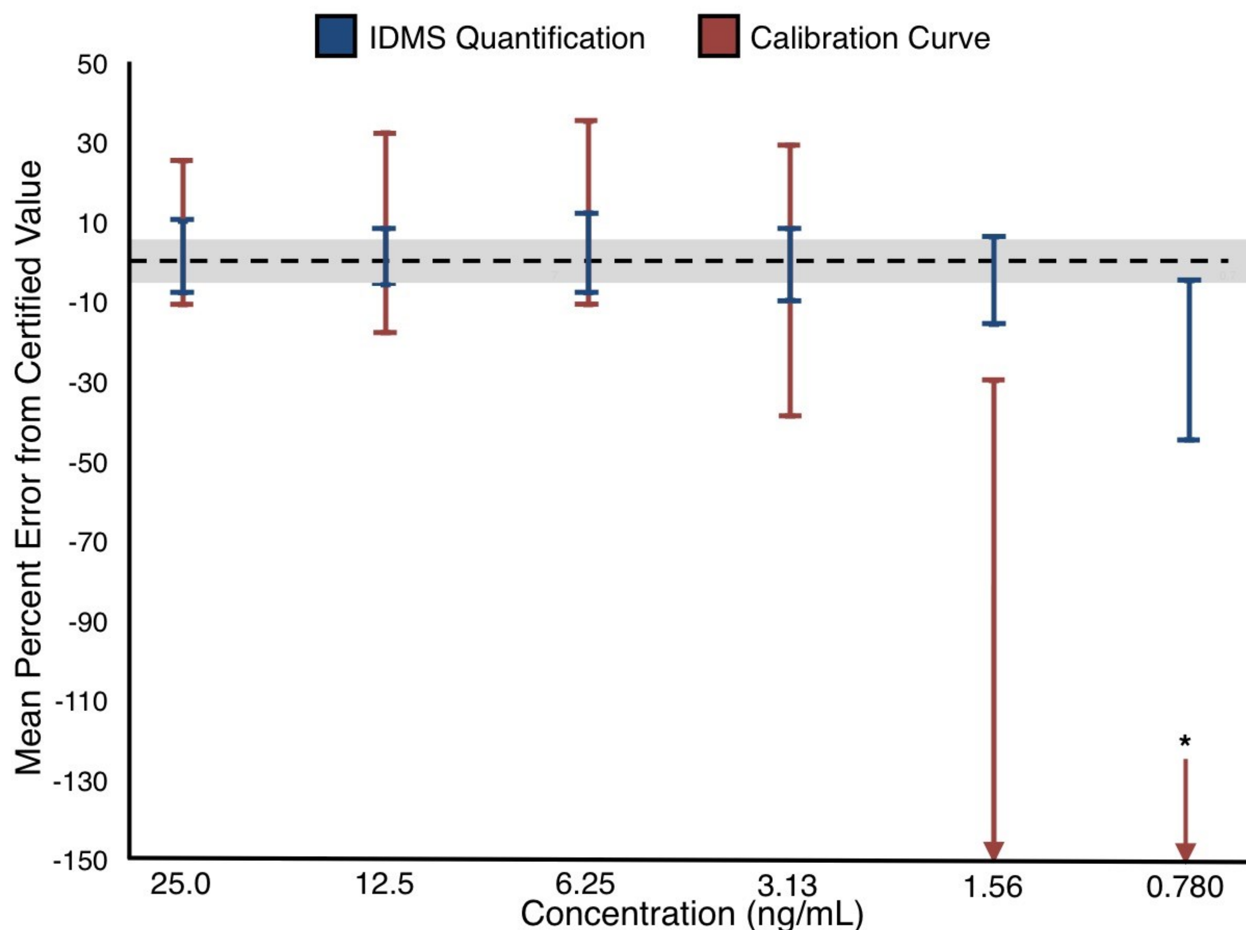

Figure A.10: A comparison of mean accuracy and precision (95% confidence range) of experimental values obtained in synthetic urine with calibration curve and IDMS across all listed analytes. Dashed black line indicates certified value and shaded area representing ±5% certified error.
*Confidence interval falls outside of viewable range

The IF+DV+AD pre-loading technique was validated for the drugs of abuse using the mixed-mode SPE column, analyzed by the optimized and validated ESI-TOF-MS instrumental method. Analysis of synthetic urine spiked with 40 ng/mL of all seven drugs of abuse using pre-loaded SPE columns 30-minutes after pre-loading produced highly accurate quantitative results.

All opioids and alkaloids with the exception of cocaine were quantified with ≤10% quantitative error and <20% RSD. Accuracy ranged from 0.766% to 13.0% error for codeine and cocaine, respectively. A mean accuracy of 5.36%±4.73% error and mean precision of 6.58%±2.92% RSD were produced by the pre-loading method. The mean experimental values obtained did not differ from the mean certified values or from the concentrations obtained using the validated ESI-TOF-MS and traditional SPE method at the 95% confidence level. Validation results for the quantification of all seven opioids and alkaloids spiked in synthetic urine and extracted using pre-loaded SPE columns 30 minutes after pre-loading can be found in table A.6.

Table A.6: Validation values for opioids and alkaloids using ESI-TOF-MS, SPE pre-loading method, and IDMS quantification in synthetic urine, showing 95% confidence

Pre-loading (n=5)	Calculated Value (ng/mL)	Experimental Value (ng/mL)	|%Error|	%RSD	LOD (ng/mL)	LOQ (ng/mL)
Morphine	40±2.00	40.6±1.61	1.59	2.93	0.429	0.8
Codeine	40±2.00	39.7±3.40	0.766	6.32	0.214	0.8
Cocaine	40±2.00	34.8±5.07	13.0	10.1	0.737	0.8
Methadone	40±2.00	44.0±6.23	10.0	10.4	0.156	0.8
6-AM	40±2.00	39.4±1.87	1.56	3.43	0.0552	0.8
Fentanyl	40±2.00	40.3±4.49	0.873	8.21	0.222	0.8
Heroin	40±2.00	36.2±1.94	9.52	3.95	0.223	0.8

Using the ESI-TOF-MS instrumental method and the IF+DV+AD pre-loading method, stability over time was assessed for the analysis of synthetic urine samples spiked with the drugs of interest. Morphine, codeine, methadone, 6-acetylmorphine, and fentanyl were quantified with adequate accuracy when analyzed one week after column pre-loading. Methadone, fentanyl, and codeine maintained adequate quantitative accuracy when quantified two weeks after column loading. At the two-week time interval, codeine maintained accuracy at the 95% confidence interval, but produced poor precision (±12%). Cocaine and heroin were not quantified accurately

after one week (15%, 17% error, respectively) or after two weeks (15%, 25% error, respectively) of storage. The stability over time results are summarized in figure A.11.

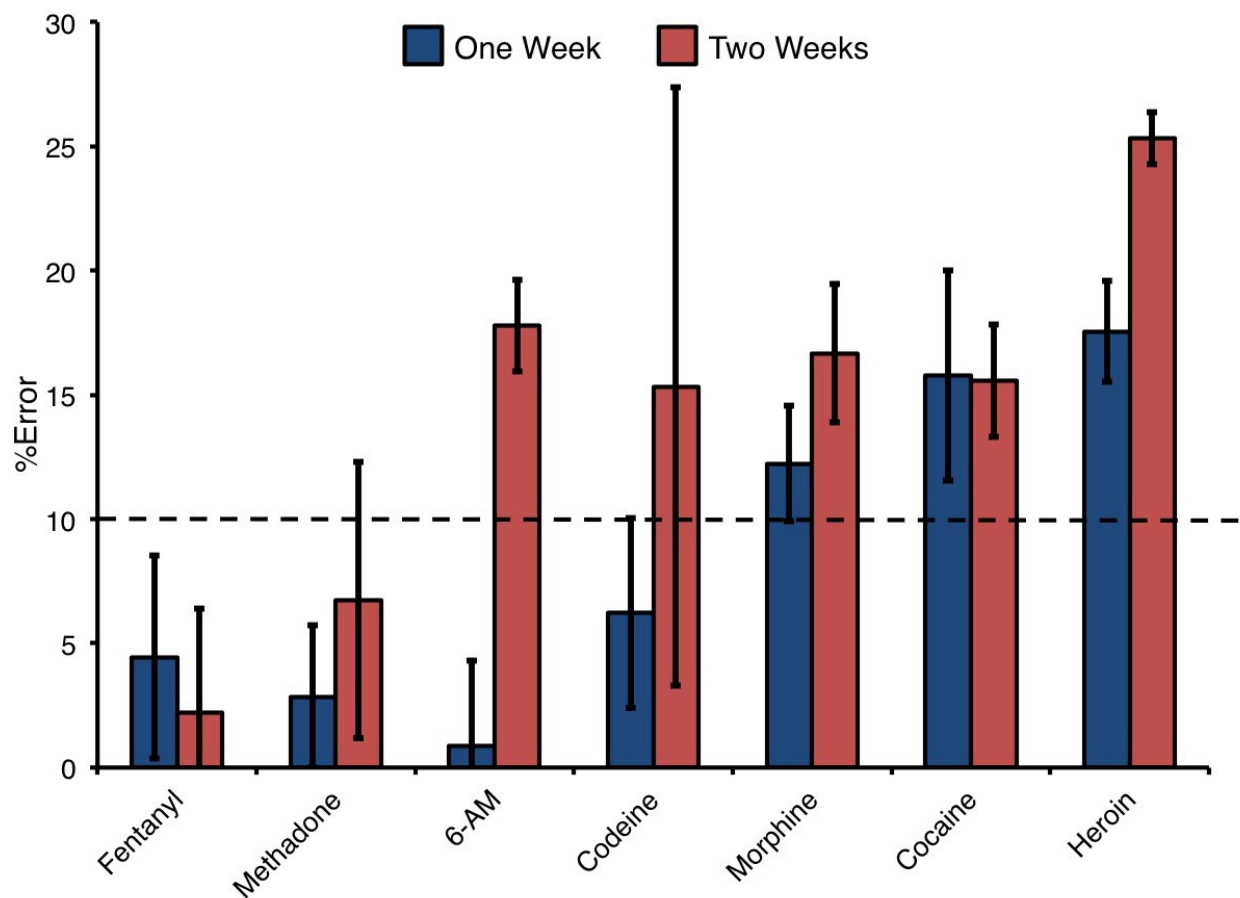

Figure A.11: Mean %error of pre-loaded columns after storage for indicated amounts of time, showing 95% confidence intervals. Dashed line represents the 10% error from certified value defined as adequate accuracy in this study.

The work presented here demonstrates the viability of isotopically pre-loaded SPE columns for the quantification of environmentally and forensically relevant analytes by IDMS. This viability was demonstrated with different SPE column packing types and a wide range of investigate analytes. While the pre-loading technique did not remain stable quantitative accuracy over time for all chosen drugs of abuse, the concept of pre-loading and storing SPE columns for future analysis has been proven viable by this research. Future work will increase the breadth and

robustness of this developed method. In subsequent research, the transfer of analytical quality using this pre-loading method will be tested in by shipment of pre-loaded SPE columns to independent laboratories for quantification of determined analytes. Providing analysts with pre-calibrated, pre-loaded SPE columns to be used for IDMS quantification may help to improve overall analytical quality.

A.4.4 Conclusions

This research has demonstrated viability of modifying SPE columns to produce pre-calibration by loading columns with accurately known concentrations of isotopically labeled analogs to remain quantitatively stable over a necessary amount of time. The method for column pre-loading was developed, optimized, and validated for quantification of the organophosphate pesticide glyphosate in drinking water. This pre-loading method was then adapted and applied to a different column packing material for the quantification of seven drugs of abuse in synthetic urine. The pre-loading method was shown to be highly quantitatively stable over a period of two weeks for glyphosate and one week for all drugs of abuse, excluding cocaine and heroin. This work is useful to researchers seeking to develop methods to improve the transfer of high accuracy and precision analytical methods between and among laboratories. Future work will be conducted on further increasing the on-column stability of analytes, increasing the breadth of applicable analytes and columns, and evaluation of field-deployment into off-site laboratories.

Appendix B: Investigation of Stir-bar Sorptive Extraction Mechanism

B.1 Introduction

The large extraction-phase volume permitted by the stir-bar design allows highly efficient analyte recovery to be achieved for appropriately hydrophobic molecules (measured by the octanol / water partition coefficient K_{ow}). The well-accepted mechanism for analyte recovery via SBSE states that analyte recovery will increase as the volume of extraction phase is increased, relative to the volume of sample. The equations describing the recovery of an analyte from an aqueous solution into a PDMS stir bar are:

$$\%Recovery = K_{o/w} / (K_{o/w} + \beta) \qquad \text{eq. B.1}$$

$$\beta = Volume_{sample} / Volume_{PDMS} \qquad \text{eq. B.2}$$

Where, $K_{o/w}$ is the octanol-water partition coefficient of a compound of interest and β is the phase-ratio between the sample and stationary phase of the extraction media. It is an accepted point in literature that by simply altering the phase-ratio by increasing the volume of PDMS in the stationary phase, a greater amount of an analyte may be recovered (when keeping all other variables unaltered). It was the hypothesis of this research, however, that describing analyte recovery from an aqueous solution by phase-ratio alone is not an accurate method of describing the extraction mechanism. This research sought to investigate the influence of surface adhesion on the total recovery of a compound from solution. It has been noted in literature that surface adsorption of analytes may play a small role in total extraction, but experiments have never verified this claim.

An environmentally relevant molecule with relatively low K_{ow} was used to explore the extraction mechanism in a quantitative manner. Three stir bars were each modified three times by removing extraction polymer, creating bars with differing surface area by identical volume compared with one another at each point. Differences in analyte recovery in the three modified stir-bars were compared with theoretical models that presumed a volume-only extraction mechanism. This experimental scheme is outlined in figure B.1.

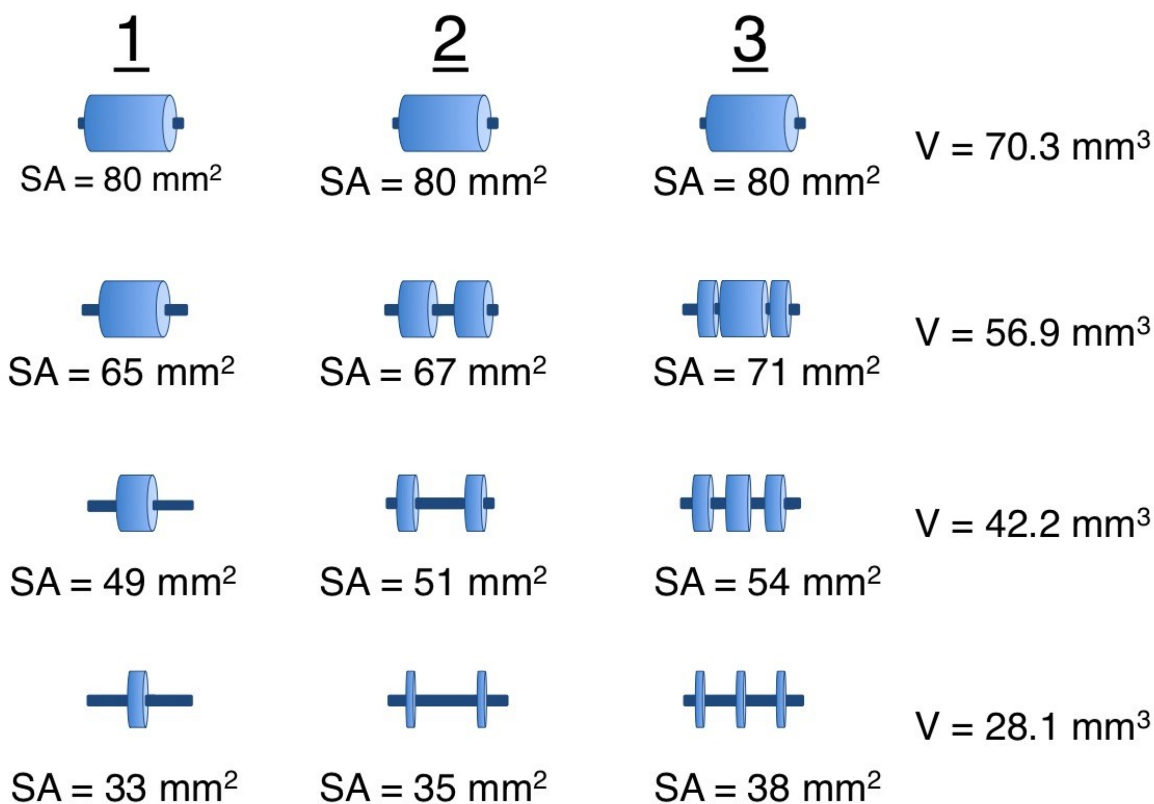

Figure B.1: Schematic of the three stir bar scenarios that produced equal PDMS volume but different PDMS surface area at four distinct points

B.2 Experimental Design

Method parameters found in Chapter 3 were used for this research. Peer-reviewed S-SBSE-IDMS methods were utilized for the quantification of naphthalene in deionized water. The

experimental design focused on minimizing variability between measurements. To obtain the most consistent and precise results, as theoretical models predicted a relatively small difference between three stir bars, care was taken to perform all method procedures identically between replicates and between stir-bars. Stir-bar extraction time was 60-minutes, measured by a stopwatch. Sample preparation was timed to assure that no dramatic differences were introduced during sample preparation. All analyte spiking and dilution procedures were conducted by mass on an analytical balance. Calculated concentration for every sample was normalized to produce a consistent value for every analysis by using the minor deviations in mass of analyte spike to correct to a standard value. Between all samples, an instrumental blank was run to ensure no carry-over effect was present. Similarly, after each analysis, every stir bar was analyzed a second time to ensure that the analytes were completely desorbed. PDMS was removed at each point using a new razor blade, weighing each bar before and after removal. New, conditioned stir-bars were used to avoid discrepancies between the surfaces of used stir-bars. Stir-bars were cleaned thermally between each replicate for 3 hours at 300 °C. Between points, following removal of PDMS, stir-bars were conditioned in solvent as recommended by the manufacturer and then thermally cleaned for 3 hours at 300 °C. All points were calculated as a "normalized mean peak area" in that the mean peak area at a given point for a given bar was plotted as a fraction of the peak area of the unmodified stir-bar.

B.3 Results

Using the extraction recovery equation, the volume of extraction phase PDMS was theoretically decreased while holding solution volume and analyte K_{ow} constant. Figure B.2 shows the two expected scenarios. Expected results gave two options: a. if the stir bars obeyed a

PDMS volume-dependent extraction mechanism, all three stir-bar modifications would produce the same recovery as PDMS was removed (black line). Or, b. if the stir-bars obeyed a surface area-dependent extraction mechanism, three distinct recovery curves would be observed (blue, red, green). Log(K_{ow}) of 3 was chosen to yield the highest quality results. Experimentally, compounds of higher K_{ow} tended to be extracted at ~100% recovery as nearly all PDMS was removed. Compounds of lower K_{ow} tended to be extracted with poor precision.

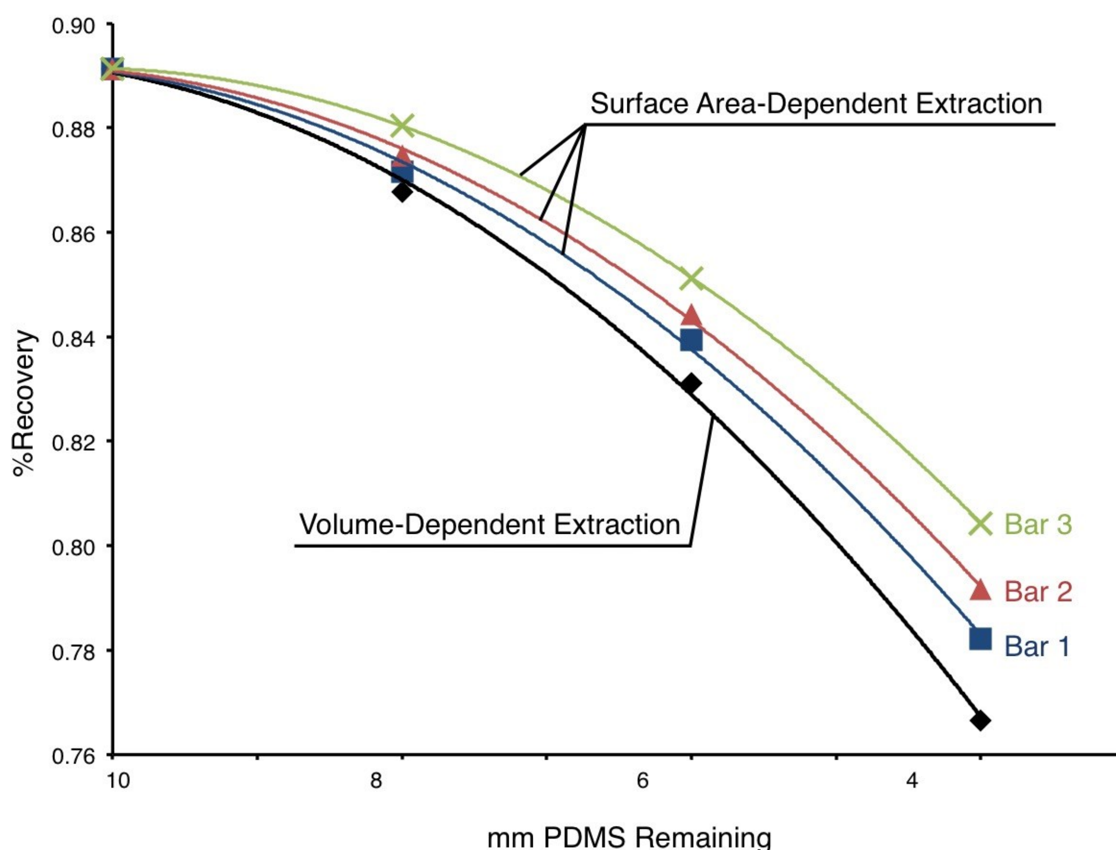

Figure B.2: Theoretical recovery of compound with log(K_{ow}) = 3 from 10 mL solution using 10mm stir bar, showing variable effect of removing increasing amounts of PDMS

Experimental results indicate that, over all PDMS-removed points, the stir bar with the least surface area extracted the most naphthalene. These results are demonstrated in figure B.3.

The extraction of naphthalene appears to have been surface area-dependent. However, the results disagree with theoretical expectations. The stir bar with the largest surface area recovered the least amount of naphthalene, all other variables held constant. N = 3 at each point and 95% confidence intervals shown.

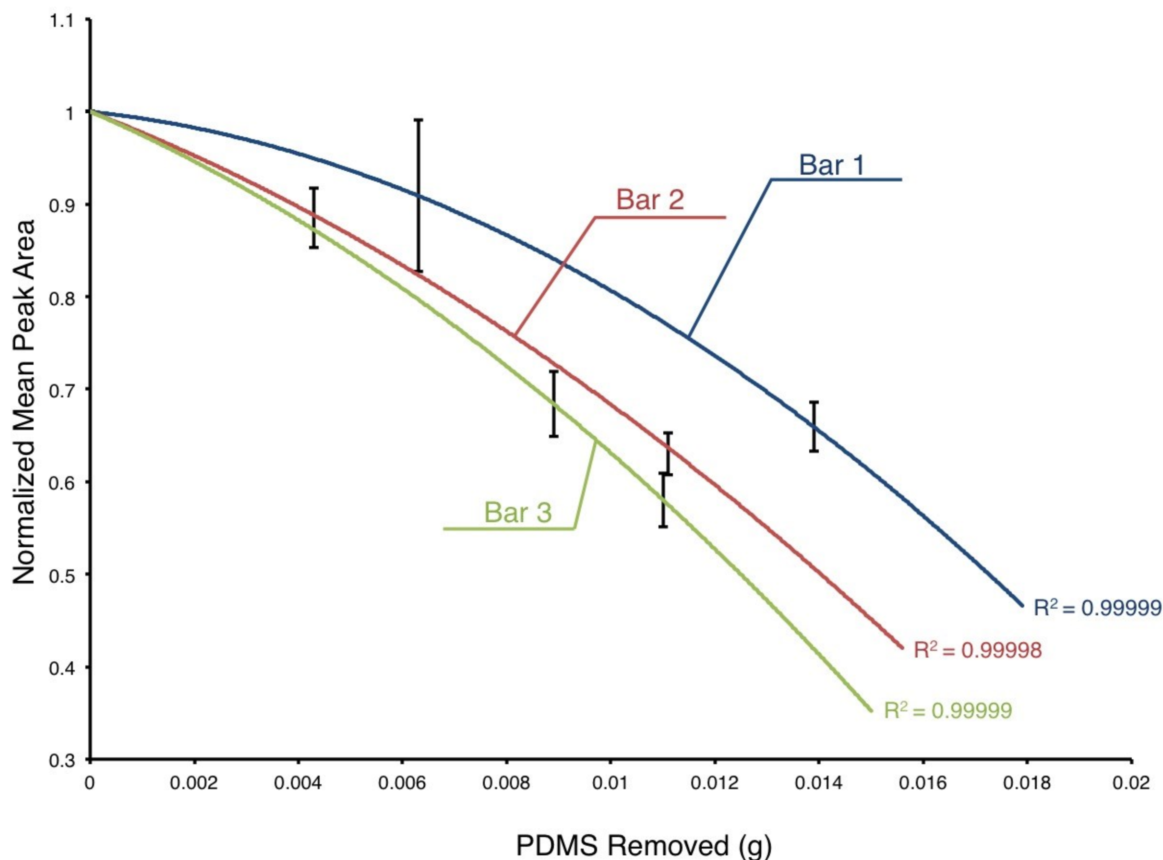

Figure B.3: Recovery trend of naphthalene from three stir bars of varying surface area as a function of mass of PDMS removed, 95% confidence shown

Comparing the amount of naphthalene recovered in stir bars with identical masses of PDMS removed produced statistically significant differences between each bar (n=3). Figure B.4 provides a focused comparison between the recovery of all three stir-bars, keeping all other variables constant. Recovery was statistically unique for each bar, with recovery decreasing with increased surface area. N = 3 and 95% confidence shown.

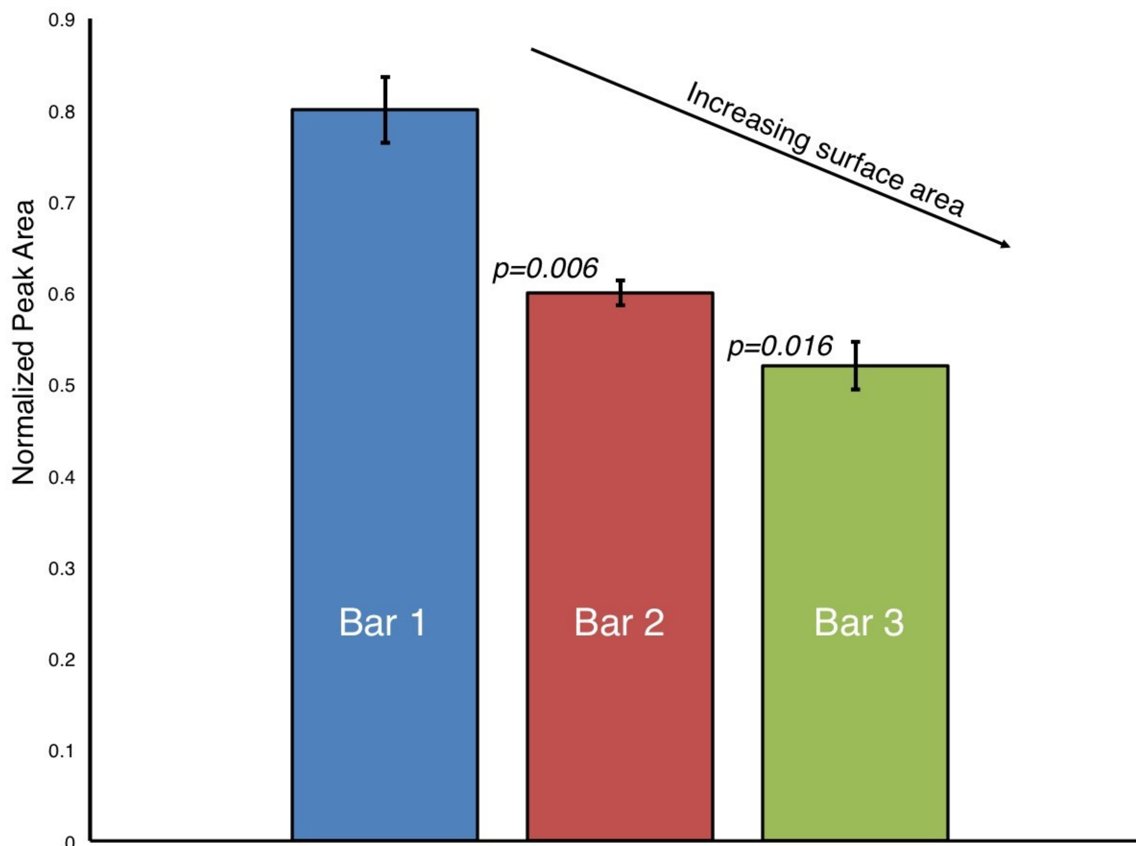

Figure B.4: Comparison of naphthalene recovery between three stir bars, at identical volumes of PDMS, 95% confidence shown

B.4 Conclusions

An experiment was conducted to investigate the mechanisms governing stir-bar sorptive extraction. It was hypothesized that increasing the surface area-to-volume ratio of a stir bar could increase the analyte recovery at extraction equilibrium. Theoretical models agreed with this, but experimental results revealed a trend between increasing surface area of a stir bar and decreasing recovery at extraction equilibrium. This effect may be explained by stir-bars with lower surface area-to-volume ratios allowing analytes to penetrate into the PDMS bulk, isolated from the solution matrix. Bars with greater surface area-to-volume ratios may permit more surface-adhesion and greater analyte-solvent contact, thereby allowing greater back-extraction into

solution. This hypothesis will be investigated in future work using laser-ablation to probe the depth of PDMS penetration.

B.5 References

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