DEVELOPMENT OF MASS SPECTROMETRY-BASED ANALYTICAL ASSAYS FOR ENVIRONMENTAL AND DEFENSE APPLICATIONS

by

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Dedicated to William Edward and Evelyn Margaret.
Find your joy in the journey.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-Amino-4-Nitroanisole	2-ANAN
2-Diethylaminoethanethiol	EDA
2-Diisopropylaminoethanethiol	IDA
2-Methoxy-5-Nitrophenol	2,5-MNP
2,4-Diaminoanisole	2,4-DAAN
2,4-Dinitroanisole	2,4-DNAN
2,4-Dintrophenol	2,4-DNP
2,4,6-Trinitrotoluene	TNT
3-((3-Cholamidopropyl) Dimethylammonio)-1-Propanesulfonate	CHAPS
3-Amino-1,2,4-Triazol-5-One	ATO
Aqueous Film Forming Foams	AFFF
Area Under the Curve	AUC
Army Research Office	ARO
Carbon Tetrachloride	CCl4
Charge to Mass of an Electron	e/m
Chemical Warfare Agent	CWA
Chemical, Biological, Radiological, Nuclear, and Explosives	CBRNE
Coefficient of Variation	%CV
Cyanoguanidine	CQ
Defense Advanced Research Projects Agency	DARPA
Desorption Electrospray Ionization	DESI
Diethyl Phosphoramidate	DEPA
Diisopropyl Methylphosphonate	DIMP
Dimethyl Methylphosphonate	DMMP
Direct Analysis in Real Time	DART
Direct Current	DC
Electron Ionization	EI
Electrospray Ionization	ESI

Ethyl Methylphosphonate	EMPA
Extracted Ion Chromatogram	EIC
Fluorotelomer Sulfonic Acid 6:2	6:2 FTS
Fourier Transform Ion Cyclotron Resonance	FT-ICR
Gas Chromatography	GC
Gas Chromatography - Mass Spectrometry	GC-MS
High Performance Liquid Chromatography	HPLC
Hydrophilic Interaction Chromatography	HILIC
Insensitive Munitions	IM
Isopropyl Methylphosphonate	IMPA
Isotopically Labeled Internal Standards	IS
Limits of Detection	LODs
Liquid Chromatography	LC
Liquid Chromatography - Mass Spectrometry	LC-MS
Liquid Chromatography Tandem Mass Spectrometry	LC-MS/MS
Mass Spectrometry	MS
Mass to Charge	m/z
Methylphosphonic Acid	MPA
N-Acetyl-L-Cysteine Methyl Ester	ACME
Nanogram	ng
Negative Ion Mode	NIM
Nitroguanidine	NQ
Nitrotriazolone	NTO
Organization for the Prohibition of Chemical Weapons	OPCW
Paper Spray	PS
Paper Spray - Mass Spectrometry	PS-MS
Paper-Based Surface Enhanced Raman Spectroscopy	pSERS
Parallel Reaction Monitoring	PRM
Part Per Billion	ppb
Part Per Million	ppm
Part Per Trillion	ppt

Partition Coefficient	Kd
Per- and Polyfluoroalkyl Substances	PFAS
Percent Relative Standard Deviations	%RSD
Perfluorodecanoic Acid	PFDA
Perfluorododecanoic Acid	PFDoA
Perfluoroheptanoic Acid	PFHpA
Perfluorononanoic Acid	PFNA
Perfluorooctane - Sulfonamide	PFOSA
Perfluorooctanesulfonic Acid	PFOS
Perfluorooctanoic Acid	PFOA
Perfluorotetradecanoic Acid	PFTeDA
Perfluorotridecanoic Acid	PFTrDA
Perfluoroundecanoic Acid	PFUdA
Pinacolyl Methylphosphonate	PinMPA
Polytetrafluoroethylene	PTFE
Positive Ion Mode	PIM
Quality Control	QC
Radio Frequency	RF
Regional Screening Levels	RSL
Reversed Phase	RP
Royal Demolition Explosive	RDX
Scientific Working Group for Forensic Toxicology	SWGTOX
Surface Enhanced Raman Spectroscopy	SERS
Tandem Mass Spectrometry	MS/MS
Total Ion Chronogram	TIC
Triple Quadrupole	QqQ
Unmanned Aerial Vehicles	UAVs
Urazole	UZ

ABSTRACT

Mass spectrometry (MS) is a powerful and versatile technique that is useful for addressing a wide range of complex analytical challenges. In this work, mass spectrometry-based assays were developed to address issues relating to environmental contamination and for detecting analytes of interest to the defense industry. Chapter one is an overview of the history of mass spectrometry, the fundamental operation of a mass spectrometer, as well as, advancements in chromatographic separation and ionization methods. Chapter two focuses on the development of an assay that uses blow flies as environmental sensors of chemical weapon release. In that work, a liquid chromatography – tandem mass spectrometry (LC-MS/MS) method was developed to detect chemical warfare agent simulants and chemical warfare agent hydrolysis products in flies exposed to the chemicals in controlled feeding experiments. The work in chapter three describes the development of a surface enhanced Raman spectroscopy assay coupled to paper spray mass spectrometry for a more fieldable and environmentally friendly approach to detect organophosphorus compounds. Chapter four describes the development of a paper spray mass spectrometry assay for the detection and semi-quantitation of per- and polyfluoroalkyl substances in whole blood without sample cleanup or chromatographic separations. This method would be useful in detecting high levels of these carcinogenic compounds in individuals highly exposed via their occupations. The final chapter (chapter five) returns to using blow flies as environmental sensors, but this time to detect insensitive munitions in the environment. The work focuses on the development of two different liquid chromatography mass spectrometry methods for the detection of insensitive munitions, which are less shock sensitive explosives, and their transformation products in the environment. Controlled feeding experiments were also performed where flies were exposed to contaminated soil and water sources to show the feasibility of this method in a more realistic scenario. The projects detailed herein show the extensive range with which mass spectrometry can be used for the detection of harmful chemistries of environmental concern.

CHAPTER 1. INTRODUCTION

1.1 History of Mass Spectrometry

Over the past century, mass spectrometry (MS) has become an indispensable analytical tool used in nearly all facets of analytical and bioanalytical chemistry. Due to its innate versatility, sensitivity/selectivity, and the ability to multiplex, mass spectrometry is often known as a "gold standard" analytical technique[1-4]. While there are many different types of mass spectrometers, a commonality between them is that all MS instrumentation analyzes and detects ions in the gas phase. Each mass spectrometer is made of an ionization source, a mass analyzer, and a detector, each of which having a critical role in the capabilities, operation, and overall function of the instrument. Although there are many combinations and permutations of the components, all mass spectrometers sort ions by their mass relative to the charge state of the ion. The reported value is known as the mass-to-charge ratio (m/z).

The field of mass spectrometry dates back to the beginning of the 20th century with J.J. Thomson who was one of the initial pioneers in mass spectrometry[5]. During Thomson's work studying cathode ray deflection in the presence of an electric and magnetic field, he discovered that he was able to indirectly measure the charge relative to the mass (e/m) of small gaseous particles, now known as electrons[6]. J.J. Thomson was awarded the 1906 Nobel Prize in Physics for this discovery. Thomson and his protégé F. W. Aston went on to build the first mass spectrometer and used it to study elemental isotope signatures, winning Aston a Nobel Prize in 1922[7-9]. In the first years of this new field, physicists utilized mass spectrometry to understand more about ions and their trajectory in magnetic and electric fields. However, it wasn't until the Manhattan Project that the usefulness and power of mass spectrometry was finally revealed[5, 10]. The calutron mass spectrometer was utilized to separate ^{235}U from ^{238}U and obtain large quantities of high-purity, enriched uranium-235, which was used to construct the first atomic bomb[11]. The sector instrument was developed in the laboratory of E. O. Lawrence from UC Berkley and operated by separating isotopes using a powerful magnetic field[11]. In the years that followed, many different types of instruments were developed, MS became more available to the masses, and significant advances in both small and large molecule characterization catapulted mass spectrometry to the forefront of analytical chemistry.

1.2 Mass Spectrometer Components

Since the initial foray into mass spectrometer development, many different types of instruments have been designed and implemented. Although their operating principles differ significantly, the three main components of mass spectrometers, the ionization source, mass analyzer, and detector, remain constant (Figure 1.1). In short, ions are generated from an ion source and then travel into the ion transfer tube of the mass spectrometer. Ion optics plates are used to guide the ions into a central beam. The mass analyzer separates and sorts ions by their m/z value. The mass analyzer is the most critical component of the mass spectrometer and is oftentimes the defining characteristic that differentiates instruments from one another. Finally, the sorted ions hit the detector, and the signals can be converted to a mass spectrum which is then relayed to the computer for analysis and interpretation.

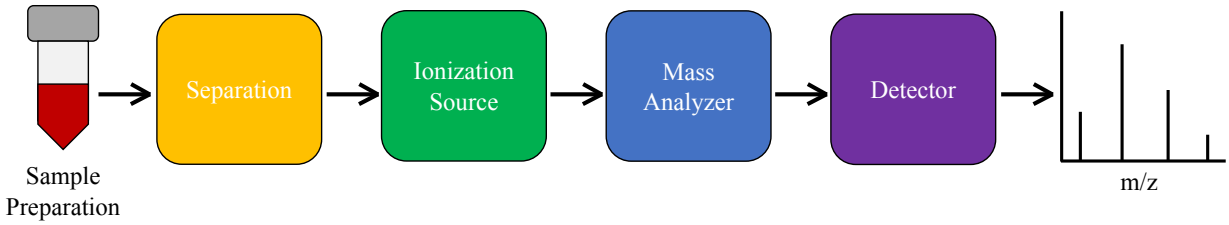

Figure 1.1 Mass spectrometry workflow overview

Mass analyzers are the critical components within a mass spectrometer used to separate ions by their mass-to-charge ratio. There have been many advancements in this area, enabling applications ranging from untargeted, exploratory experiments to part-per-trillion (ppt) level quantitation. Mass analyzers can be separated into two major categories, low- and high-resolution instruments[12]. Low resolution instruments such as the ion trap, quadrupole, and sector analyzers can only measure a m/z out to two decimal places making separating contaminants and analytes at the same nominal mass nearly impossible. Although low resolution instruments are lacking in specificity, they make up for it in decreased cost and higher throughput. Additionally, adding a fragmentation step (MS/MS) oftentimes overcomes the limitation of background or contaminant peaks. High resolution instruments such as orbitrap, time-of-flight, and Fourier transform ion cyclotron resonance (FT-ICR) instruments are often necessary when analyzing macromolecules or doing untargeted/unknown analysis due to their resolving power (>100,000) and mass accuracy

(<1 ppm). While they do provide many advantages in comparison to low-resolution instruments, they are costly to purchase/maintain and require more bench space.

Two mass analyzers were employed in the work in this dissertation: quadrupole and orbitrap mass analyzers. While a quadrupole can be used alone, three quadrupoles in tandem, known as a triple quadrupole (QqQ) instrument, are utilized frequently in high throughput applications, such as for quantitative toxicology assays. Each quadrupole has four cylindrical rods (Fig. 1.2A), where radio frequency (RF) voltages and direct current (DC) voltages are applied. Opposite rods have the same polarity, which results in an oscillating electric field between the rods. As ions are transmitted through the quadrupole, the electric field can be modified so that only ions with a certain m/z value have a stable trajectory, resulting in mass selection[13]. In a QqQ instrument, the first quadrupole is used for mass selecting a precursor ion that can then be fragmented using inert gas molecules in the second quadrupole. The fragments are then mass selected in the third quadrupole for detection. The high-resolution instrument (Q-Exactive Focus) utilized in this dissertation also contains a quadrupole to mass select precursor ions that can then be detected by the high resolution orbitrap (Fig. 1.2B). The orbitrap was invented by Makarov in the 1990s and was commercialized by Thermo Scientific in 2005[14]. In this type of mass analyzer, ions orbit around a central spindle electrode. Ions are separated based on the frequency with which they oscillate around the central electrode. Fragmentation can also be performed by mass selecting a precursor ion in the quadrupole and initiating a collision with inert gas molecules in a collision cell. From there, the ions are injected into the orbitrap via the C-Trap and fragment and precursor ions can be detected simultaneously.

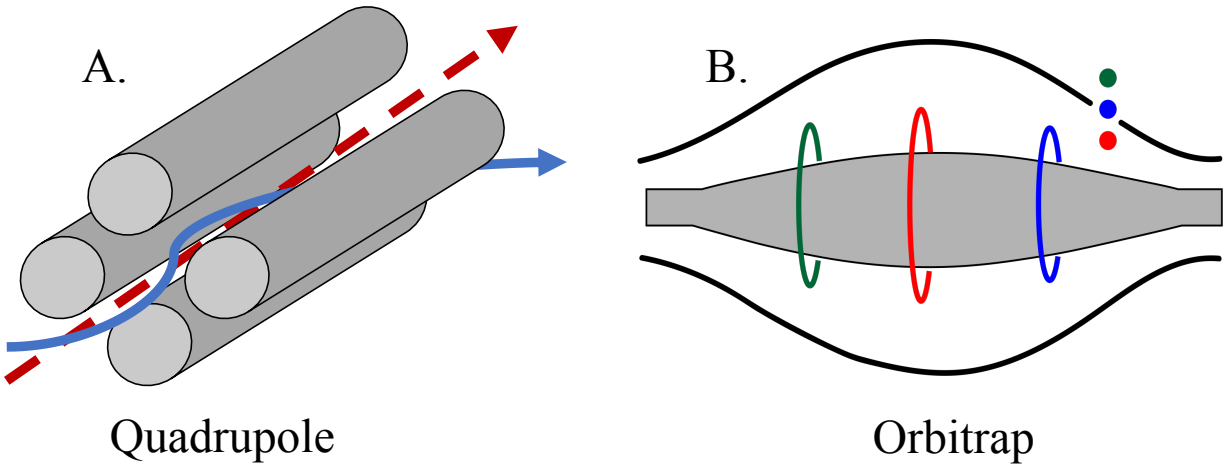

Figure 1.2. Schematic of a quadrupole mass analyzer (A) and an orbitrap mass analyzer (B). In the quadrupole, at a particular RF and DC voltage ions that are stable (red dash line) are allowed to pass through the mass filter whereas unstable ions are ejected (blue line). In the orbitrap, ions orbit around a central spindle electrode and are separated based on their mass to charge.

1.3 Ionization Fundamentals

While the mass analyzer is important, the ionization source selected for an application is critical because it determines the type of ions that are generated and detected by the MS. There are two broad categories, hard and soft ionization sources which differ by the amount of energy imposed upon a molecule during ionization. The most common hard ionization source is electron ionization (EI). EI ionizes molecules by passing the volatilized compounds near a wire filament producing electrons with a kinetic energy of 70 eV[12]. The electrons cause the molecules to eject an electron, making them positively charged with virtually the same mass as the unionized molecule. However, EI not only ionize the molecule of interest, but oftentimes causes it to fragment. Fortunately, the fragmentation is very reproducible and a library of fragmentation patterns is often utilized to "match" the mass spectra to a known reference material. EI is widely utilized when coupled to gas chromatography (GC) for detection and quantitation of volatile and semi-volatile compounds. Alternatively, soft ionization methods, such as electrospray ionization (ESI), do not cause the precursor compound to fragment, leaving the pseudo-molecular ion peak intact. In ESI, a sample in solution is passed through a needle with an applied voltage (2-5 kV), which creates a potential difference between the needle and the inlet of the mass spectrometer[15]. The potential difference causes the solvent flowing through the needle to spray in charged droplets

towards inlet, which is known as a Taylor cone[16]. As the solvated analytes travels from the needle to the mass spectrometer, the solvent evaporates and the charged droplets become smaller and smaller until they reach the Rayleigh limit, where the charge is too much for the total volume of the droplet which makes the droplet unstable[17]. Once this occurs, the droplet undergoes fission into many smaller droplets. Eventually the solvent evaporates entirely leaving the charged analyte in the gas phase to then enter the MS. Unlike EI, ESI produces a pseudo molecular ion peak that differs from the molecular mass. In positive mode, proton addition typically occurs creating an $[M+H]^+$ ion, whereas in negative ion mode proton loss occurs forming $[M-H]^-$ ions. While other adduct ions do occur, it is analyte and condition dependent. Two of the main benefits of ESI is that the pseudo molecular ion can be used for identification purposes and the samples must be in solution, overcoming the two major pitfalls of EI. ESI allows for labile compounds and larger molecules such as proteins to be analyzed by MS.

1.4 Liquid Chromatography

It was the advent of ESI that allowed for liquid chromatography couple to mass spectrometry (LC-MS) to flourish. Up until then, gas chromatography was the primary chromatographic technique able to be coupled to mass spectrometry (GC-MS) due to the sample being volatilized in the column prior to the ionization step. The key to gas chromatography, however, is that the analytes of interest must be somewhat volatile. Many large molecules, such as large biomolecules, are labile and cannot be analyzed by GC-MS. Liquid chromatography (LC) is a powerful analytical tool that separates analytes based on their affinity for the mobile and stationary phases. There are four major categories for LC separations, reverse phase, normal phase, ion exchange and size exclusion chromatography, that differ by their stationary and mobile phase composition, as well as their retention modes (Figure 1.2)[18]. Reverse phase chromatography (Fig. 1.2 A) is the most common retention mechanism. It relies on a hydrophobic stationary phase (typically C8 or C18) and a gradient from an aqueous solvent to an organic solvent. Hydrophobic analytes are retained by the stationary phase in the presence of high-water content in the starting mobile phase. The hydrophobic interactions are disrupted as the mobile phase increases in organic solvent. In contrast to reverse phase, normal phase has a polar stationary phase (typically silica-based) and a nonpolar mobile phase (Fig. 1.2 B). Normal phase is used to separate polar analytes that would otherwise be unretained on a reverse phase column. Analytes that remain charged at a

variety of pHs require a different mode of retention. The third mechanism (Fig. 1.2 C) is known as ion-exchange, and oppositely charged analytes are retained on a charged stationary phase. There are two main types of ion exchange chromatography, cation and anion exchange. Fig. 1.2 C shows an anion exchange mechanism, where the stationary phase is positively charged. Analytes are eluted by increasing the ionic strength in the mobile phase, displacing the analytes of interest, and causing them to elute. The final main mechanism of retention is size exclusion chromatography (Fig. 1.2 D). This mechanism is utilized to separate analytes by their size. This technique is often used when separating large biomolecules or polymers. The column is filled with porous filtration beads that act as the stationary phase. Larger analytes will elute faster because they move around the large beads, while smaller analytes slow down as they travel through the pores. There are other modalities as well, known as affinity chromatography, hydrophilic interaction chromatography (HILIC), and chiral chromatography, which rely on similar principals mentioned here[18].

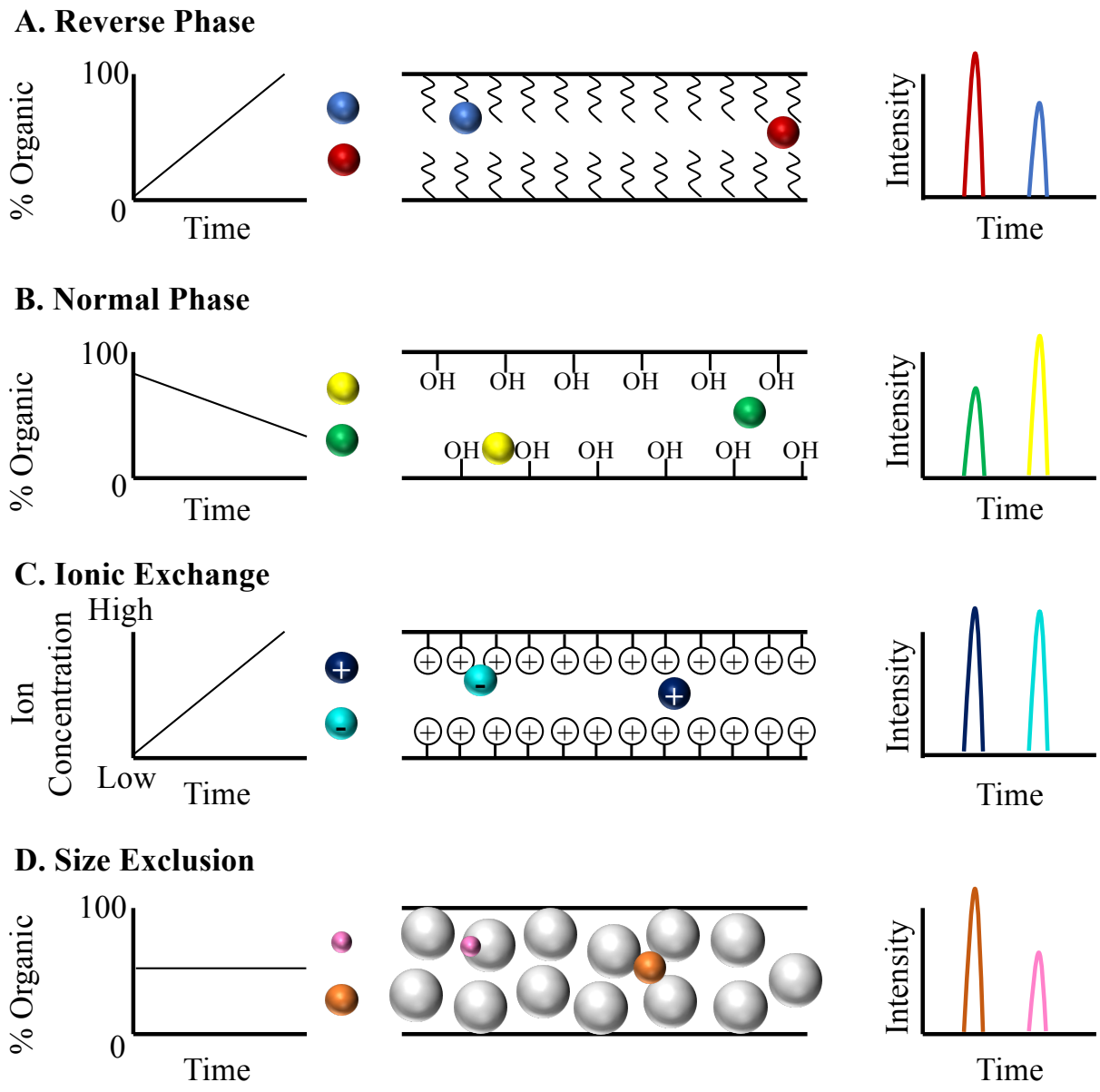

Figure 1.3 Liquid chromatography retention mechanisms

1.5 Ambient Ionization

While many industries and applications rely heavily on chromatographic-based separations prior to mass spectral analysis, chromatography is labor intensive, expensive, and increases time per sample. Since the early 2000s there has been a heavy focus on the development of ambient ionization methods that provide alternatives for sample introduction. The first techniques developed were desorption electrospray ionization (DESI)[19] and direct analysis in real time

(DART) in the early 2000s. In these two techniques, samples are placed near the ion transfer tube of the mass spectrometer. In DESI, electrosprayed solvent is used to desorb the analyte ions from a surface to then enter the MS inlet. DART, on the other hand, uses ionized gas molecules directed towards a sample to ionize the analyte molecules which will then enter the MS. Since then, there has been an explosion of ambient techniques that continues to this day[20]. Due to the innate limitations of ambient methods such as matrix effects, low recovery, and lower specificity, oftentimes high resolution or tandem MS is required to improve confidence in the measurements.

Two projects in this work utilize the ambient ionization technique known as paper spray (PS) ionization for rapid detection of analytes relative to protecting the warfighter and civilians. PS was initially developed by the R. Graham Cooks and Zheng Ouyang groups at Purdue University in the 2010s[21-24]. Traditionally in PS-MS, a small (5-10 μL) drop of biofluid is spotted onto a piece of chromatography paper cut to a sharp point (Figure 1.3). A spray solvent (100-200 μL) is applied to the paper substrate along with a voltage that induces an electrospray-like event at the tip of the paper. The resulting plume is known as a Taylor cone and contains droplets of analyte molecules dissolved in solvent. Similar to ESI, as the charged droplets travel toward the MS inlet, the solvent evaporates leaving charged analyte molecules in the gas phase. As soon as the voltage is applied, the analyte signal increases and stays constant until the solvent is depleted or the voltage is turned off. In this technique, there is no chromatographic separation. The resulting signal over time is therefore known as a chronogram. PS-MS provides an alternative to traditional LC-MS assays that is simple, rapid (~1 minute) and can handle complex sample matrices such as whole blood or soil without any sample cleanup. Since its advent, this technique has been utilized in many clinical[22-32], environmental[33-38], forensic applications[27, 39-56], and has even been commercialized. QuantIon Technologies, Inc developed the first commercial prototypes for an automated paper spray source and disposable cartridge. The technology was later transferred to Prosolia, Inc. (Indianapolis, IN), which adapted the prototypes into commercially available instrument called the Velox. Additionally, Thermo Scientific has developed an automated paper spray module called the VeriSpray that can be attached to their triple quadrupole mass spectrometers. The VeriSpray has been used for drug checking facilities[55], quantitating Remdesivir in patient samples[57], sports drug testing[58], and paper spray method optimization studies[59], as well as others.

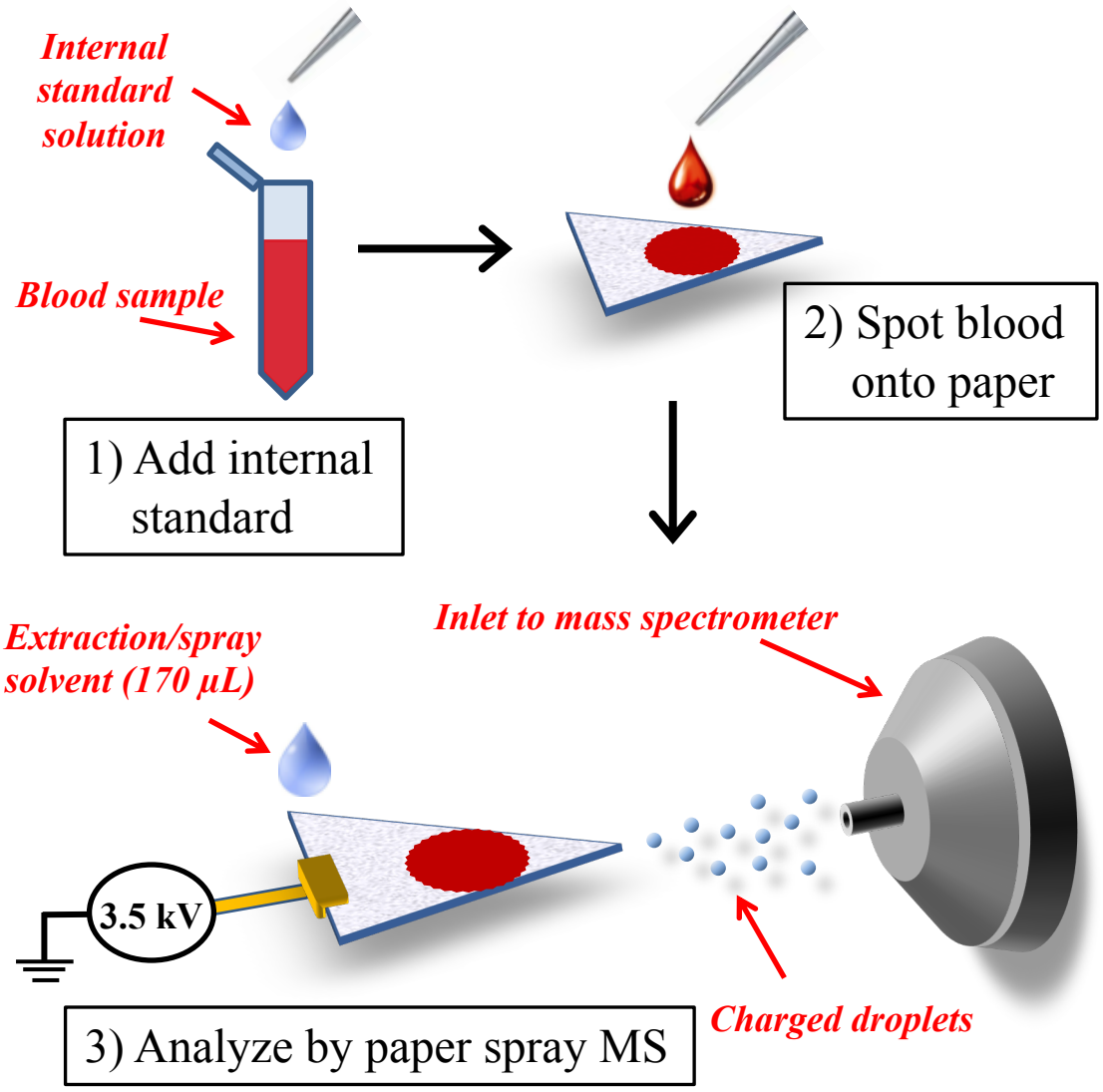

Figure 1.4. Traditional paper spray mass spectrometry (PS-MS) workflow.
Figure from Shi, et. al. [29]

The work described within this dissertation describes the development of several mass spectrometry assays to address applications in the chemical defense and military arenas. Not only is it useful when the goal is to protect civilians from dangerous chemical releases, but to also monitor sworn personnel for potential line-of-duty exposure. This work aims to address gaps in the current methodologies regarding three key areas: the detection of chemical warfare simulants and hydrolysis products (Chapters 2 and 3), semi-quantitation of per- and polyfluoroalkyl substances (PFAS) in human whole blood (Chapter 4), and environmental contamination of insensitive munitions (Chapter 5).

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CHAPTER 2. INSECTS AS CHEMICAL SENSORS: DETECTION OF CHEMICAL WARFARE AGENT SIMULANTS AND HYDROLYSIS PRODUCTS IN THE BLOW FLY USING LC-MS/MS

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2.1 Abstract

In this work, blow flies are investigated as environmental chemical sample collectors following a chemical warfare agent (CWA) attack. Blow flies sample the environment as they search for water and food sources and can be trapped from kilometers away using baited traps. Three species of blow flies were exposed to CWA simulants to determine the persistence and detectability of these compounds under varying environmental conditions. A liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed to detect CWA simulants and hydrolysis products from fly guts. Flies were exposed to the CWA simulants dimethyl methylphosphonate and diethyl phosphoramidate as well as the pesticide dichlorvos, followed by treatment dependent temperature and humidity conditions. Flies were sacrificed at intervals within a 14-day post-exposure period. Fly guts were extracted and analyzed with the LC-MS/MS method. The amount of CWA simulant in fly guts decreased with time following exposure, but were detectable 14 days following exposure, giving a long window of detectability. In addition to analysis of CWA simulants, isopropyl methylphosphonic acid, the hydrolysis product of sarin, was also detected in blow flies 14 days post-exposure. This work demonstrates the potential to obtain valuable samples from remote or access-restricted areas without risking lives.

2.2 Introduction

Chemical warfare agents (CWAs) are highly toxic chemicals that are dispersed as aerosols, deposited on surfaces, or are released in the environment[1, 2]. These dangerous chemistries cause harm through incapacitation often resulting in lethal effects[3]. CWAs have a long and tempestuous history that culminated in the creation of the Chemical Weapons Convention in 1992[1, 4]. This treaty outlaws the production, stockpiling, or use of chemical weapons. Although many nations around the world have signed the treaty thereby agreeing to reduce their disclosed stockpiles of chemical weapons, concerns remain that rogue nations and extremist fringe groups maintain or can produce unknown amounts of CWAs. This poses a great risk to nations around the world. There have been a few recent instances of chemical weapons attacks by both state and non-state forces. Between 2012 and 2019, there have been over 300 instances of chemical weapons attacks during the Syrian Civil War[5-8]. In 2018, the nerve agent VX was used in the assassination of Kim Jong-nam[9, 10]. In November 2019, following the Salisbury/Amesbury incidents, the Novichok agent A-234 was added to the Chemical Weapons Convention's list of controlled substances[11, 12]. This was the first chemical added since the treaty was agreed upon in the 1990s[13].

New, innovative sample collection and detection methods is needed to help address the continued use of chemical warfare agents. Attacks warranting an investigation by the Organization for the Prohibition of Chemical Weapons (OPCW), such as in Syria, require multi-national cooperation and costly long-term investigations. By the time samples are finally collected and analyzed at a qualified laboratory, trace amounts of the intact agent remain, leaving behind hydrolysis products[14, 15]. It is essential to be able to rapidly detect these chemicals to determine appropriate actions following a suspected CWA attack. While research has shown success in detecting chemical and biological warfare agents in the environment, these sample collection methodologies still require humans to enter the potentially contaminated area[15-21]. This requirement not only potentially exposes responders to the toxins, but also slows the overall response in the field.

In addition to their use as weapons, organophosphorus molecules have a long history of environmental contamination and impact[22]. These molecules have been used in agriculture since the 1960s as pesticides to prevent crop damage from insects[23]. Approximately 40% of pesticides used around the world are organophosphorus molecules[23]. Due to their popularity, the

environmental impact of these chemicals has been studied at length and exposure to these chemicals can happen via foodstuffs through ingestion or dermal absorption[24, 25]. In addition to pesticide usage, old organophosphorus munitions have been disposed of in the environment resulting in ecosystem alterations at the dump sites[26]. These alterations can result in contaminated foodstuffs, similarly to pesticide usage. Following the discharge of a CWA, through disposal or intentional release, there is a need to prevent exposure to the warfighter but also secondary exposure to civilians through the environment.

Fortunately, there have been advancements in remote and passive sample collection and subsequent analyses. For example, unmanned aerial vehicles (UAVs) and drone technology are at the forefront of hands-off sample collection[27, 28]. However, these utilize complex instrumentation that are overt in nature and risk discovery. Additionally, their cumbersome shape limits accessibility in rough terrain, restricting their use. Biological species such as *Tradescantia[29]*, honeybees (*Apis mellifera L.*)[30], fruits, and vegetables[31, 32] have been monitored for pesticide exposure. In addition, honeybees have been trained and deployed to detect explosives[33]. The ideal sample collection method for CWAs would be covert, rapid, easy to use, inexpensive, and support sensitive detection, which thus far has yet to be developed.

This project proposes a solution by utilizing blow flies as environmental sample collectors and sensors. Blow flies constantly sample their environment via fluid acquisition from carrion[34], feces[35], vegetation[36-38], and standing water. Moreover, they are found in a variety of climates and ecosystems on every continent except Antarctica[39]. Blow flies are commonly used by forensic entomologists to accurately estimate portions of the postmortem interval (i.e., the time elapsed since death, PMI)[40-43]. In addition to forensic entomology, blow flies are powerful indicators of vertebrate resource diversity[44]. Blow flies can be used to inexpensively and indirectly monitor changes in animal communities by analyzing the stomach contents for animal DNA[45-47] and chemical signatures of animal feces[48], as well as by performing stable isotope analysis on fly heads to determine previous carcass resources in the environment[49].

Flies can harbor multitudes of important biological and chemical information just by living in and sampling their environment. Chemical analysis of blow flies can therefore be used for chemical sampling of dangerous or remote regions[50]. Catch-and-release experiments with *Phormia regina* showed that flies can be recovered from 13 to 45 kilometers away using baited traps[34]. Blow flies have the capabilities of traveling large distances; *P. regina* was recorded up

to 45km from its dispersal point[51]. When important resources are plentiful, however, blow flies tend to stay in the immediate vicinity and not travel away from valuable reproductive opportunities[52]. Flies seem to better detect baits placed upwind relative to their location, but they also can locate a bait when no wind is detected in the local environment[53]. Collection of blow flies therefore has the potential to provide personnel with valuable samples from targeted areas without risking the lives of chemical, biological, radiological, nuclear, and explosives (CRBNE) teams, warfighters, or other responders. The goal of this project was to develop analytical techniques to detect CWA simulants and CWA hydrolysis products in fly gut contents as well as to develop a preliminary understanding of the longevity of these agents in flies using controlled feeding experiments.

2.3 Experimental Methods

2.3.1 Chemicals and Materials

High-performance liquid chromatography (HPLC) grade methanol, HPLC grade water, and ammonium acetate were purchased form Fisher Scientific (Hampton, NH, USA). Chemical warfare simulants: dimethyl methylphosphonate (DMMP), diethyl phosphoramidate (DEPA) and diisopropyl methylphosphonate (DIMP), hydrolysis products: ethyl methylphosphonate (EMPA), isopropyl methylphosphonate (IMPA), and pinacolyl methylphosphonate (PinMPA), and organophosphorus pesticides: dichlorvos, malathion, and methyl parathion were all purchased from Sigma Aldrich (St. Louis, MO, USA). The isotopically labelled internal standard d_7-IMPA was purchased from Sigma Aldrich and $^{13}Cd_3$ - DIMP internal standard was obtained from Dr. Bob Williams and Mark Alvarez at the Los Alamos National Laboratory (Los Alamos, NM, USA).

2.3.2 Calibrator and QC Preparation

Working solutions were prepared in methanol from the certified reference materials and were frozen for later use. Calibrators were serially diluted from working solutions in water. The final concentration range for the chemical warfare simulants and pesticides calibrators was 15.6 ng/mL to 2000 ng/mL. The final concentration range for hydrolysis products was 3.9 ng/mL to 500 ng/mL. A separate set of working solutions were made for the quality control (QC) samples. QC samples were prepared at low, medium, and high concentrations within the dynamic range.

An internal standard solution was prepared by diluting reference materials in methanol to 2000 ng/mL for the stable isotope labeled chemical warfare simulants/pesticides and 500 ng/mL for the hydrolysis products. Prior to analysis, internal standard (7.5 μL) was spiked into calibrators and QCs (200 μL). Due to the semi-quantitative nature of this study, calibrators and QCs were analyzed in neat solutions and were not matrix matched.

2.3.3 Blow Fly Extraction

Experimental flies were freeze-killed and portions of the digestive system were dissected out with flame-sterilized forceps. The insect digestive system consists of a foregut (including the crop and proventriculus), a midgut, and a hindgut[54]. For the purposes of this study, the foregut and midgut were dissected for chemical analysis and will henceforth be referenced to as the "guts". Chemical compounds of the guts were extracted by sonicating in 100 μL of methanol for 30 minutes followed by nitrogen evaporation and reconstitution in 200 μL of water to improve retention of the analytes. All flies were extracted separately and were analyzed individually. The internal standard mix was spiked into the reconstituted sample.

2.3.4 LC-MS/MS Assay Development and Validation

Assay development and validation were performed on an UltiMate 3000 HPLC system and a Q-Exactive Focus mass spectrometer from Thermo Fisher Scientific (San Jose, CA, USA). A Hypersil GOLD C18 (100 mm x 2.1 mm, 3 μm particle size) analytical column and a Javelin guard column was used for analysis. A 10 μL injection volume was used, and the autosampler needle was washed before and after all sample injections. Column temperature was maintained at 40°C with an autosampler temperature at 4°C. The mobile phase consisted of 5mM ammonium acetate (A) and methanol (B). The gradient was run at a flow rate of 0.2 mL/min as follows: 0-1.5 min hold at 2%B, 1.5-3.5 min linear ramp from 2% to 95% B, 3.5-7 hold at 95%B, then ending with a re-equilibration period of 6 minutes at 2% B making for a 13-minute method.

Table 2.1 Analytes included in HPLC-MS/MS method validation. The table includes a description of the compound, the collision energy, the MS transition, and the deuterated standard utilized for each analyte.

Analyte	Description	Polarity (+/-)	CE (V)	Transition (m/z)	Retention Time (min)	Internal Standard
EMPA $[M-H]^-$	MPA	-	12	123.0 → 94.9904	1.42	IMPA-d_7
IMPA $[M-H]^-$	MPA	-	14	137.0 → 94.9904	2.03	IMPA-d_7
DMMP $[M+H]^+$	Simulant	+	15	125.0 → 111.0205	4.71	DIMP[C_{13}]-d_3
DEPA $[M+H]^+$	Simulant	+	12	154.1 → 98.0005	6.11	DIMP[C_{13}]-d_3
PinMPA $[M-H]^-$	MPA	-	21	179.1 → 94.9904	6.88	IMPA-d_7
Dichlorvos $[M+H]^+$	Pesticide	+	14	220.9 → 127.0154	7.1	DIMP[C_{13}]-d_3
DIMP $[M+H]^+$	Simulant	+	20	181.1 → 97.0051	7.11	DIMP[C_{13}]-d_3
Methyl parathion $[M+H]^+$	Pesticide	+	15	264.0 → 142.9924	7.47	DIMP[C_{13}]-d_3
Malathion $[M+H]^+$	Pesticide	+	12	331.0 → 127.0391	7.54	DIMP[C_{13}]-d_3
DIMP[C_{13}]-d_3 $[M+H]^+$	IS	+	13	185.1 → 101.0273	7.11	
IMPA-d_7 $[M-H]^-$	IS	-	14	144.0 → 94.9904	2.03	

Following LC separation, the samples were analyzed using the Q-Exactive Focus mass spectrometer with the following parameters: 20 sheath gas (arbitrary units), 320 °C ion transfer tube temperature, the S-lens set to 50 V, and a 35,000 resolution. As previously described, the MS was calibrated in both positive and negative ion mode at least once within a 7-day period[21]. Mass spectral data were acquired using parallel reaction monitoring (PRM) with MS/MS fragmentation, with an isolation window of ±0.5 *m/z* in both positive (4 kV) and negative ion

mode (3 kV) depending on the analyte. An inclusion list was used with windows around the analytes' retention times to facilitate the correct polarity. Polarity switching was utilized around 7 minutes because of the close elution of PinMPA (negative ion) and dichlorvos (positive ion). A list of the analyte MS conditions, including transitions used for quantitation, can be found in Table 2.1.

2.3.5 Internal Validation Procedures

The validation procedures followed guidelines proposed by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology guidelines[55]. Briefly, eight-point calibration curves were made using serially diluted stock solutions. The concentration range was between 15.625 – 2000 ng/mL for the compounds analyzed in positive ion mode and 3.9 - 500 ng/mL for the compounds analyzed in negative ion mode. These calibration curves were run over the course of 6 days along with separately prepared quality control samples. We also evaluated the inter-day variability of the method by calculating the bias and %CV over 6 days for the three internally prepared QCs. Blanks with internal standard and double blanks were run to show selectivity and specificity.

2.3.6 Assessment of Short-Term Stability and Degradation

The stability of the QCs was evaluated at each level during the validation to determine the impact of storage conditions on the analysis. The middle level, QC 2, was stored under three temperature conditions (−20 °C, 4 °C, and 22 °C) and aliquots were analyzed at 0, 24, 48 and 72 hours. The QCs were run in duplicate for each storage condition. The freeze-thaw stability of QC 2 was evaluated by freezing 3 aliquots at -20°C. All aliquots were removed from the freezer and re-frozen at each time point during the study over the course of the 72-hour time period culminating a total of 3 freeze-thaw cycles for the final analysis.

A 10-day degradation study was performed to assess the stability of the DMMP, Dichlorvos, and DEPA over time. High, medium and low QCs were aliquoted and analyzed on 0, 1, 3, 5, 7, and 10 days at room temperature using the validated method. The area under the curve (AUC) was compared to the frozen standards on the day of analysis.

2.3.7 Recovery

Extraction recovery was estimated by spiking blank fly guts with a known quantity of the analytes. The guts were allowed to dry and then extracted as described above. Recovery was calculated as the ratio of fly gut spiked before extraction to fly gut extract spiked after the extraction process relative to the internal standard signals.

2.3.8 Detection of Transformation Products

Hydrolysis of the chemical warfare agent simulant DIMP was studied to assess the likelihood of detecting transformation products, in addition to the fed chemical, in the fly after exposure. A benchtop experiment was performed where a 1 mg/mL solution of DIMP was acidified with formic acid (0.1%) and heated at 80 °C for an 8-hour time period. Aliquots were sampled at 0, 0.5, 1, 2, 4, and 8 hours and were diluted in water to 1000 ng/mL. A 1 mg/mL solution of DIMP was also fed to the flies and the signal of DIMP and IMPA was monitored in the fly gut over the 14-day period.

2.3.9 Blow Fly Colony Formation

Wild adult blow flies were collected from a local urban park (Military Park, Indianapolis, IN, USA; 39.770555, -86.168611) using a rancid chicken liver bait and an aerial sweep net. Live flies were anesthetized by placement in a refrigerator set at approximately 4˚C for 30 min and morphologically identified to species using a dichotomous key[56]. Species of interest were sorted and placed into their respective cages, representing generation zero (G_0) of each laboratory colony. Blow fly species used in this study included the black blow fly *Phormia regina* (Meigen), the secondary screwworm *Cochliomyia macellaria* (Fabricius), and the common green bottle fly *Lucilia sericata* (Meigen). Approximately 200 to 500 adults comprised each G_0 colony.

After formation of each colony, oviposition (i.e., egg-laying) was encouraged by introducing approximately 20g chicken liver along with a chicken blood-soaked Kimwipe (Kimberly-Clark Global Sales, Inc., Roswell, GA, USA) and a distilled water-soaked Kimwipe within an 88.7 mL white plastic cup to each cage. Exposure to this resource lasted approximately 18 h and continued daily for 3 – 4 d. Rearing containers consisting of 946 mL mason jars, were made each day to house cohorts of eggs and growing larvae. Each jar contained an 88.7 mL bath

cup with ~50g chicken liver inoculated with 100 – 200 eggs which sat on top of a pupation substrate of soft pine bedding. Each jar was topped with a breathable Wypall disposable paper cloth (Kimberly-Clark Global Sales, LLC, Roswell, GA, USA) secured by the jar ring. Larvae were allowed to develop in the jar until adult emergence was observed. Adults of each species were then integrated into their respective colonies. To ensure that large colonies were maintained throughout the duration of this project, protein-feeding and oviposition induction of adults took place every three to five days. Additional wild collections were performed throughout the study period to supplement the colonies. For each species, there existed a maintenance colony (to continue propagating individuals for the next generation) and an experimental colony. All adults and larvae of laboratory colonies were maintained under ambient conditions (~22˚C, 50%RH, 12:12 L:D cycle) in the "fly room" at IUPUI.

2.3.10 Feeding Experiments

All feeding experiments followed the same general design. All flies used for experiments were $<G_{10}$. For each experiment, an equal number of three- to four-day old male and female adult flies were randomly selected from the experimental colony. These flies were placed in a sterilized cage and only exposed to water and sugar. Each fly was individually placed inside of a clean 37.0 mL plastic portion cup (Carolina Biological Supply Company, Burlington, NC, USA) containing a 2.54cm^2 Kimwipe soaked in 100 µL of either a CWA simulant or distilled water (negative controls). Exposure lasted 4 h under ambient conditions in a chemical fume hood. Following exposure, flies were placed into treatment or negative control cages and given water and sugar *ad libitum*. Flies were maintained under a 12:12 L:D cycle, with treatment dependent temperature and humidity conditions, in a Percival I-36VL incubator (Percival Scientific Inc., Perry, IA, USA). Flies were killed via freezing at -20˚C at different time intervals post-exposure: 0h, 1d, 3d, 5d, 7d, 10d, and 14d. At each sample collection time, an equal number of male and female flies were removed from each of the treatment and control cages and freeze-killed until further use.

Experiment 1: CWA Variation. Cohorts of N = 6 *P. regina* (3 male, 3 female) were exposed to either 0.0 mg/mL (negative control) or 1.0 mg/mL of either DMMP or DEPA and maintained post-exposure at 25˚C and 60%RH.

Experiment 2: Temperature Variation. Cohorts of N = 6 *P. regina* (3 male, 3 female) were exposed to 0.0 mg/mL (negative control) or 1.0 mg/mL DMMP and maintained post-exposure at five different temperatures: 17, 20, 25, 30, and 35˚C. Percent RH was maintained at 60% for all temperatures.

Experiment 3: Relative Humidity Variation. Cohorts of N = 6 *P. regina* (3 male, 3 female) were exposed to 1.0 mg/mL DMMP and maintained post-exposure at four different relative humidity settings: 25, 40, 60, and 80%RH. Temperature was maintained at 25˚C across all humidity treatments.

Experiment 4: Variation in Blow Fly Species. Cohorts of N = 6 flies (3 male, 3 female) from two additional blow fly species (*C. macellaria* and *L. sericata*) were exposed to either 0.0 mg/mL (negative control) or 1.0 mg/mL DMMP and maintained post-exposure at 25˚C and 60%RH.

Experiment 5: Lethality of Dichlorvos. Cohorts of N = 12 *P. regina* (6 male, 6 female) were exposed to six concentrations of Dichlorvos: 0.0 mg/mL (negative control), 0.01, 0.1, 1.0, 5.0, and 10.0 mg/mL. All flies died within 1.5 h of exposure.

Experiment 6: DIMP Transformation Product. Cohorts of N = 6 *P. regina* (3 male, 3 female) were exposed to either 0.0 mg/mL (negative control) or 1.0 mg/mL of DIMP and maintained post-exposure at 25˚C and 60%RH.

2.3.11 Data Analysis

Tracefinder version 3.3 from ThermoFisher Scientific (San Jose, CA) was used to analyze the LC-MS/MS data. Calibration curves were prepared using the ratio of the analyte AUC to the internal standard AUC; the line of best fit was determined using 1/x weighted linear least squares regression[57]. The LOD was estimated by multiplying the quotient of the standard error of the y-intercept and the slope by a factor of 3.

2.4 Results and Discussion

2.4.1 Method Validation

Figure 2.1 shows an overlaid chromatogram of the analytes included in the study. Two of the three alkyl methylphosphonic acid hydrolysis products eluted within the first 3 minutes of the method, which was expected due to their high water solubility. DMMP eluted towards the middle of the analytical method. The remaining analytes required higher organic content in the mobile phase before eluting from the column. Although there were some co-eluting peaks, adequate selectivity was still achieved because both the precursor ion and the MS/MS fragmentation of the compounds were different.

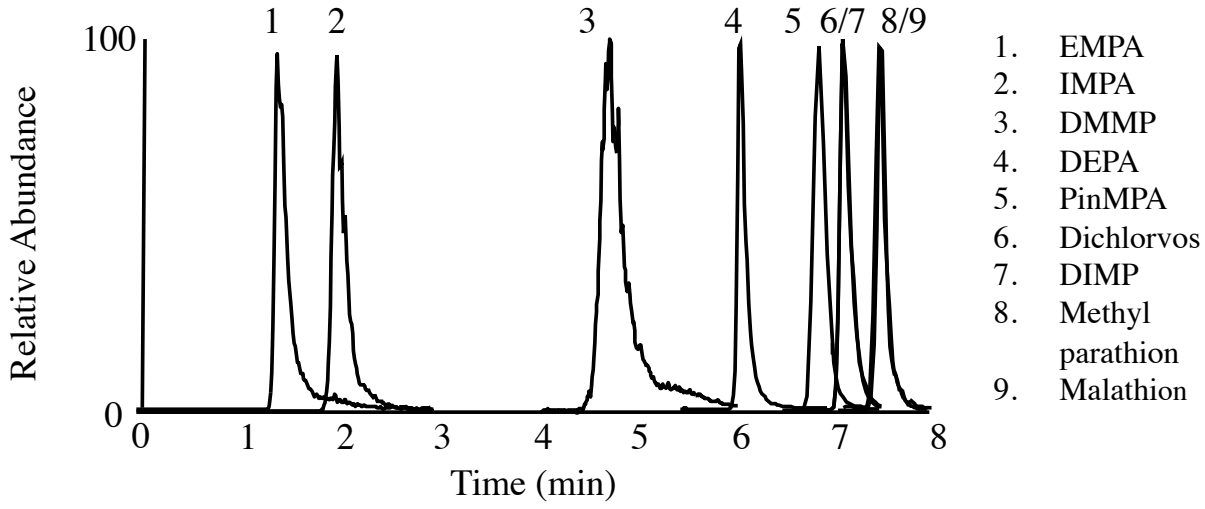

Figure 2.1 Overlayed extracted ion chromatograms of the analytes measured in the LC-MS/MS method

The aim of this assay was to measure approximate amounts of the simulants and hydrolysis products in fly guts. Accurate and precise quantitation was not possible because the sample amount (fly gut) could not be exactly known; the mass of an individual fly gut was variable but too small to weigh. Moreover, the fluid intake by the blow flies, even during the controlled feeding experiments performed here, is expected to vary from fly to fly. General trends can therefore be elucidated from the analytical data, but quantitative concentrations cannot be determined from individual measurements.

With these inherent limitations in quantitation in mind, the linearity, accuracy, precision, limit of detection, and stability was assessed according to SWGTOX guidelines[55]. Table 2.2 shows the detection limits and performance for the calibration curves over the six-day validation. The detection limits were found to be in the low ng range. These amounts are the mass of analyte in the 100 µL methanol extract and therefore represent the amount detectable in the fly gut. Table 2.3 details the inter-day variation of the quality control response at each level. In a traditional toxicology LC validation, the %CV and bias should be no higher than 15%. The vast majority of the values from the validation fell around 15%. The recovery of the extraction protocol ranged from 49% for the pesticide dichlorvos and to a high of 87% for the G-series simulant DIMP (Table 2.3). This quantitative performance was considered adequate for our application.

To evaluate the stability of the neat calibrators, analyte signal was assessed at various temperature conditions and over three freeze-thaw cycles. The analytical signal between QC 2 aliquots at these different conditions was not deemed statistically significant ($p > 0.16$) for all compounds, in all conditions. These results indicated that samples could be stored either in the freezer, chilled autosampler, or benchtop prior to analysis and could be freeze-thawed without degradation risk. In addition, QCs were monitored when frozen and stored on the benchtop with the same sampling frequency as the feeding experiments.

Table 2.2 The average LODs, range of LODs, and the range of coefficient of determinations (R^2) obtained across the six-day validation.

Compound	Avg. LOD (ng)	LOD Range (ng)	r^2 Range
EMPA	0.12	0.02 - 0.28	0.9907 – 0.9987
IMPA	0.12	0.08 – 0.3	0.9714 – 0.9976
DMMP	0.72	0.3 – 1.34	0.9464 – 0.9920
DEPA	0.9	0.22 – 2.2	0.9724 – 0.9939
PinMPA	0.28	0.16 – 0.62	0.8982 – 0.9908
Dichlorvos	0.6	0.22 – 1.26	0.9706 – 0.9936
DIMP	0.72	0.14 – 2.24	0.9733 – 0.9970
Methyl Parathion	0.66	0.3 – 1.42	0.9546 – 0.9894
Malathion	0.62	0.1 – 1.78	0.9840 – 0.9985

Table 2.3 Inter-day bias and precision values over the course of the six-day validation period. %Bias= (grand mean of calculated concentration-nominal concentration/nominal concentration). %CV=standard deviation/mean. % Recovery was determined only at the high.

	Low QC		Medium QC		High QC		
	% CV	Bias	% CV	Bias	% CV	Bias	% Recovery
EMPA	5	-7	4	-13	8	-9	52
IMPA	13	-13	19	-19	5	-12	56
DMMP	4	4	8	-17	9	7	59
DEPA	19	-6	14	17	14	-4	72
Dichlorvos	3	3	5	-20	8	1	49
DIMP	6	-4	3	-2	12	1	82
PinMPA	9	6	13	-26	7	-38	52
Malathion	9	-14	20	-22	7	-11	55
Methyl parathion	22	-17	15	-27	14	1	58

2.4.2 Feeding Studies

Controlled feeding experiments were performed in which DMMP and DEPA were fed to *P. regina* under controlled environmental conditions. The amount and detectability of the two simulants in individual blow flies are shown in Figure 2.2A. In general, the amount of simulant decreased as the time since exposure increased. The decreased amount measured in the fly is likely due to metabolism and excretion. Strikingly, both DEPA and DMMP were frequently detectable 14 days after fly exposure, which could give a long window for detection of fly exposure to chemical agents and hydrolysis products. DMMP clearance rate was found to depend on environmental conditions. Figure 2.2B shows a decrease in the detectability of DMMP at higher temperatures for 7-14 days post-exposure; we hypothesize that the higher temperature increased the fly metabolic rate. A similar result, albeit less pronounced, was also observed for humidity (Figure 2.2C). Finally, two additional species of blow fly, *C. macellaria* and *L. sericata*, were exposed to DMMP along with *P. regina* to determine if additional species of blow fly could be used as environmental sensors. Detection of DMMP, both in terms of the amount and clearance, was similar in *C. macellaria* and *L. sericata* compared to *P. regina*. Samples were not collected for Day 14 of this experiment.

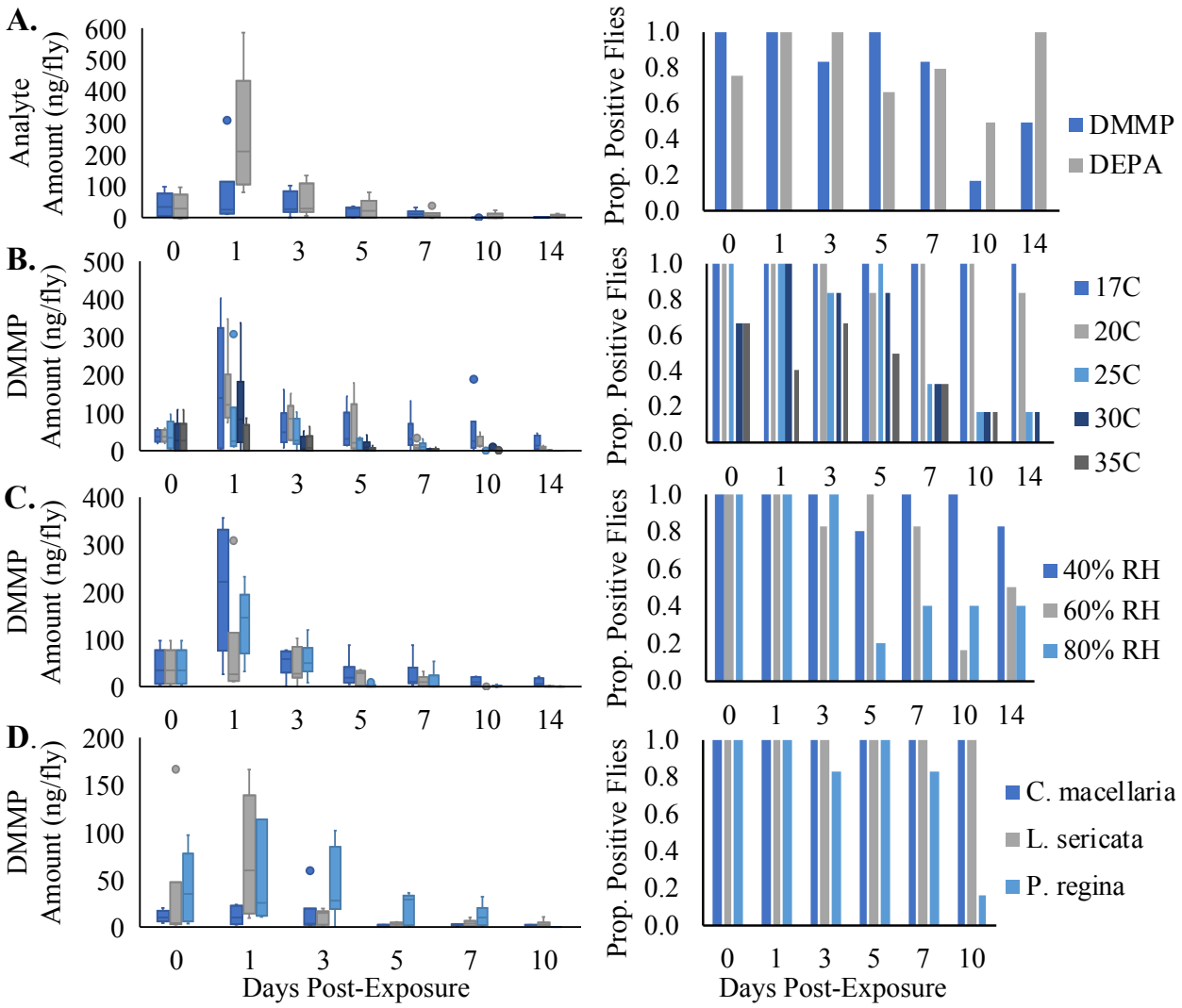

Figure 2.2 Results from the feeding experiments. The left column shows box-and-whisker plots plotting the analyte amount detected in blow fly guts at each day post-exposure. The right column shows the proportion of flies in the cohort with detectable analyte sign.

It should be noted that there are cases where the proportion of positive flies is higher later in the study. For example, Figure 2.2C shows 50% of the flies were positive for DEPA on Day 10 whereas 100% were positive on Day 14. Unlike traditional biofluid assays where a single patient is monitored over a given time period, different flies were sacrificed and dissected at each time interval. Therefore, there is innate variability in the volume of water consumed by each individual fly as well as variable metabolism.

Feeding experiments were also performed for dichlorvos, which, unlike DMMP and DEPA, is a toxic pesticide. All exposure concentrations (ranging from 0.01 to 5.0 mg/mL) resulted in

deaths of all flies, but with no detectable dichlorvos signal in the guts of the flies. Dichlorvos is used as both a contact insecticide and as a fumigant[58]. Blow flies presumably died from either exposure to fumes or from contact with the solution prior to any consumption. The heads, guts, and whole bodies of dichlorvos-exposed flies (1 mg/mL) were extracted and analyzed to determine the detectability of dichlorvos in other parts of the fly. Dichlorvos was detected in 2/10 heads and 8/10 whole flies, indicating that the flies died due to inhalation and/or physical contact with the chemical, not via ingestion. One implication of this finding is that blow flies may be killed on contact with CWA, and therefore would not be trapped remotely. Future work utilizing actual chemical agents will need to be carried out to determine if chemical agents can be detected in flies after nonfatal exposures.

2.4.3 Detection of CWA Transformation Product

Considering the toxicity of G and V-series CWAs, exposure to the intact agents, depending on the concentration, would be fatal to blow flies. If the insects are killed or their motility significantly reduced, they obviously cannot be trapped remotely using bait. However, nerve agents readily hydrolyze in the environment to give nontoxic organophophonic acids[59]. The rate of hydrolysis is pH and temperature dependent. Sarin, for example, hydrolyzes to form IMPA with a half-life of 24h at pH 7.4 and 25°C[60] while soman hydrolyzes to PinMPA with a half-life of 9.6 hours at pH 7.6 and 30°C[61]. In the actual environment, degradation could be slower if the nerve agents are sequestered in organic-rich compartments away from water. Significant amounts can remain for weeks[60]. Nevertheless, we hypothesize that flies entering a contaminated area at some point after chemical agent release may be exposed to significant amounts of hydrolysis product rather than the intact CWA. EMPA, IMPA, and PinMPA, which are hydrolysis products of VX, sarin, and soman, respectively, were included in the LC-MS/MS method for this purpose. Data on the persistence of the hydrolysis in environmental samples limited. EMPA was found to have a half-life of 8 days[62] while Baygildiev found IMPA and PinMPA soil concentrations in the low μg/g range at a chemical weapons plant that had been decommissioned for 25 years[19]. Methylphosphonic acid is stable in the environment[60].

To investigate the detection of a CWA transformation product, we selected the simulant DIMP for further investigation. Preliminary experiments indicated that DIMP hydrolyzed in the blow fly into IMPA, the same hydrolysis product formed by sarin. To test for *in vitro* hydrolysis,

aqueous solutions of DIMP were prepared at pH 7 and 2.7 (to simulate the pH of the blow fly midgut[63]) and heated for 8 hours. The area under the curve of DIMP was plotted over the 8-hour period (Figure 2.3A). No degradation of DIMP was observed under either condition. A feeding experiment was also performed in which the flies were fed 1 mg/mL concentrations of DIMP (Figure 2.3B). Interestingly, DIMP was not detected in any of the flies in this study even at the 0-day time point (4 hours after exposure). However, all of the flies had detectable signal for the hydrolysis product IMPA. Because the benchtop experiment showed no DIMP hydrolysis even with heat and acidification, the formation of IMPA in the fly suggests some rapid *in vivo* metabolic process rather than simple hydrolysis in the acidic environment of the fly gut. These results indicate that even if the parent CWA is not detected in the fly, metabolic transformation or hydrolysis products could still be detectable for days after exposure. Therefore, both the target molecules and their hydrolysis products should be analyzed when utilizing blow flies for environmental chemical detection.

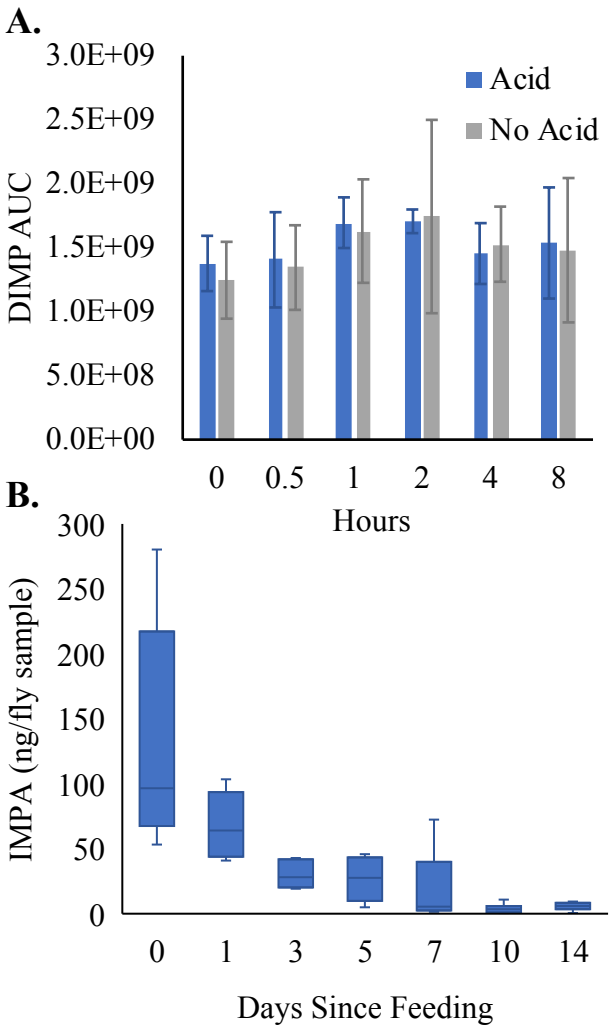

Figure 2.3 (A) Benchtop degradation experiment showing no DIMP degradation after heated and acidified. (B) Box-and-whisker plot showing IMPA amount detected in the flies after exposure to DIMP.

2.5 Conclusion

The experimental data presented in this work shows the potential for blow flies to be used as indicators of CWA dispersal in the environment. A 13-minute LC-MS/MS assay was developed and validated to detect nine chemical warfare agent simulants, chemical warfare hydrolysis products, and organophosphate insecticides. This LC-MS/MS method was used to monitor the CWA simulants DMMP and DEPA in flies up to 14 days post-exposure in a variety of environmental conditions. In addition, this work has shown the ability to not only detect CWA simulants but also CWA hydrolysis products in the flies. Future work is needed to make the

analysis more field-friendly, which will include testing fieldable instrumentation and a simpler extraction protocol using whole flies. Research is also needed to establish detectability of actual chemical nerve agents in realistic scenarios, though work with these agents requires special precautions and regulatory approvals. Overall, our method shows promise in using a wild, untrained organism to naturally acquire important chemical information from its environment while minimizing physical harm to frontline workers.

2.6 Acknowledgements

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CHAPTER 3. DUAL-TECHNIQUE ASSAY FOR ANALYSIS OF ORGANOPHOSPHORUS COMPOUNDS FOR ENVIRONMENTAL AND CHEMICAL DEFENSE APPLICATIONS

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3.1 Abstract

Forensic and environmental sciences often rely on chromatographic separations coupled to mass spectrometry to detect contaminants in complex matrices. However, these methods require lengthy analysis times and sample preparation that is not suitable for analysis in the field. In this work, two analytical methods were combined that are known for their potential for portable analysis. The ambient ionization technique, paper spray mass spectrometry (PS-MS) was coupled to paper-based surface enhanced Raman spectroscopy (pSERS) to detect toxic organophosphorus molecules from the same substrate, with a total analysis time of less than five minutes. The coupling of these techniques presents a potential for portable Raman screening followed by MS confirmation in a field-forward laboratory. A cartridge insert was designed and 3D printed to facilitate the sample collection and analysis for PS-MS and pSERS. Three chemical warfare agent simulants: dimethyl methylphosphonate (DMMP), diethyl phosphoramidate (DEPA), and diisopropyl methylphosphonate (DIMP) were included in the method due to having similar chemistries to G- and V-series chemical warfare agents (CWAs). Organophosphorus pesticides, malathion and dichlorvos, with similar mechanisms of action to the CWAs, were also included in the method. Because CWAs quickly degrade in the environment, the CWA hydrolysis products, ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PinMPA), methylphosphonic acid (MPA), 2-Diethylaminoethanethiol (EDA), and 2-Diisopropylaminoethanethiol (IDA) were also studied. A mixture of the analytes was used to create calibration curves using the dual-polarity, PS-MS method with sub-ng to low ng limits of detection. A dilution series, spanning 3 orders of magnitude, was made using pSERS, also with low ng limits of detection. These experiments show the potential and feasibility for PS-

MS coupled to pSERS to be used to rapidly, screen and confirm the presence of organophosphorus molecules, in complex matrices, with portable instrumentation.

3.2 Introduction

Mass spectrometry (MS)-based assays are the quintessential analytical methods for clinical, environmental, and forensic fields[1-4]. However, mass spectrometry assays traditionally rely on chromatographic separation prior to MS analysis, and these techniques are associated with lengthy analysis times, complex sample preparation, bulky instrumentation, and require rigorous maintenance to continue operation. These factors make traditional mass spectrometry assays impractical for in situ analysis. Researchers over the last 10+ years have addressed these concerns in one of two ways: (1) Make the instrumentation more field-friendly through miniaturization of the mass spectrometer[5-11]; or (2) Simplify the sample preparation step by using ambient ionization techniques to eliminate the chromatographic separation[12-20]. The focus of this paper is on the latter approach.

Following the development of desorption electrospray ionization (DESI)[19] and direct analysis in real time (DART)[21] in 2004-2005, a multitude of ambient ionization techniques have emerged that are alternatives to traditional ionization mechanisms. One of the most popular techniques developed was paper spray ionization. First developed in 2010, paper spray MS works by applying a sample in solution to a piece of chromatography paper cut to a sharp point[12]. Prior to MS analysis, solvent is applied to extract the analytes of interest. After the application of a voltage, an electrospray-like event occurs at the tip of the paper. Paper spray and its previous applications have been reviewed in McBride et. al[22] and Cardozo da Silva et. al[23].

Raman spectroscopy is a compelling option for pairing with mass spectrometry. It is nondestructive and gives orthogonal information with MS. However, due to the inherent inefficiency of inelastic photon scattering, classical Raman experiments cannot be used for trace analysis[24]. Research over the last 30+ years has focused on developing a solution to this issue through Surface Enhanced Raman Spectroscopy (SERS)[24-33]. SERS increases the intensity of inelastically scattered photons. Enhancement arises due to increased local electric field strength at the surface of plasmonic particles. Molecules in close proximity to the surface exhibit significantly enhanced Raman sensitivities. A wide variety of substrates have been developed for SERS analysis[34]. The vast majority of SERS applications, however, utilize materials fabricated

in the research laboratory and have not been commercially produced. In this study, commercially available paper-based SERS (pSERS) substrates were used, making the method more attractive to practitioners looking to adopt these methods.

In addition to the rapid and field friendly nature of these two analytical techniques, they provide a greener analytical methodology that is beneficial for both cost effectiveness and reducing overall waste production. Neither analytical method requires any sample preparation or extraction, and the only solvent required for the assay is a spray solvent (120 μL) which is entirely consumed during the paper spray method. The only waste being produced during a PS-MS assay is the paper tip. The pSERS substrates are biopsy punched into 3 mm disks in order to analyze more samples per strip reducing overall costs. The pSERS and paper spray methods described in this work operate in tandem to rapidly pre-screen samples to reduce the burden on an analytical laboratory.

Although the coupling of these analytical methods is fairly new, researchers have recently proven the feasibility and usefulness of the pairing. Since 2017, Patrick Fedick and coworkers have published three papers coupling SERS and paper spray. The first paper published in 2017 gave the first description of the combination and gave an overview of applications[30]. The second, focused specifically on fentanyl and fentanyl analogs, used both a portable Raman and portable mass spectrometer[35]. The most recent paper took a different approach and designed a laboratory experiment for undergraduate chemistry students, showing that individuals other than experienced researchers can learn and execute these analytical methods[36]. In addition, Christopher Mulligan and coworkers showed how using SERS in combination with paper spray MS can be used to differentiate isomers that were indistinguishable by MS or MS/MS[37]. Finally, Christopher Gill and coworkers used paper spray and SERS (separately) for point-of-care illicit drug checking and overdose prevention[38, 39].

In this work, paper spray MS and pSERS were used to detect toxic organophosphorus molecules from the same substrate. The rapid, field-friendly, and nondestructive nature of pSERS makes it amenable as a screening method prior to confirmation by MS. Commercially available pSERS substrates were used with a fieldable Raman spectrometer. An automated, plate-based paper spray source coupled to a triple quadrupole MS was used to perform MS analysis from the SERS paper for additional confirmation. In addition, 3D printed devices were developed to facilitate a seamless transfer between the analytical methods. A proof-of-concept experiment was

performed to show the potential for these two analytical methods to be used for surface detection via wiping (Figure 3.1).

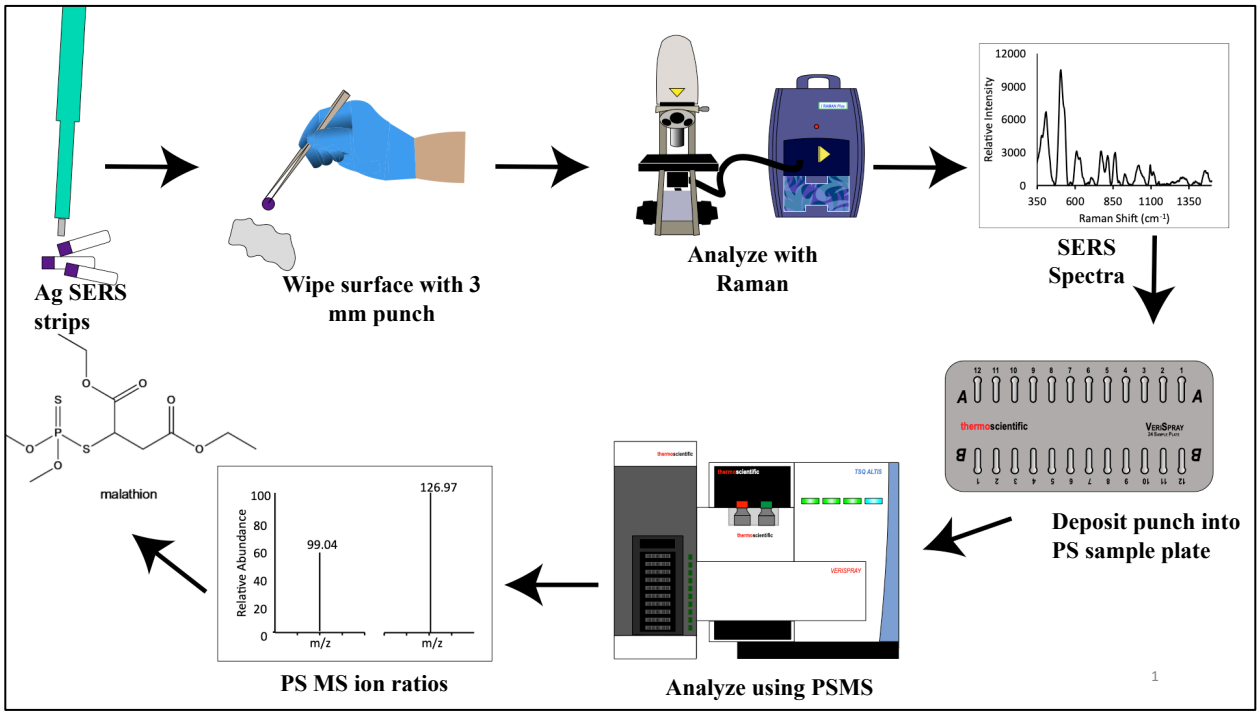

Figure 3.1 SERS to PS sample analysis workflow

3.3 Experimental Methods

3.3.1 Chemicals and Materials

High-performance liquid chromatography (HPLC) grade methanol, HPLC grade water, and formic acid were purchased form Fisher Scientific (Hampton, NH, USA). Chemical warfare simulants: dimethyl methylphosphonate (DMMP), diethyl phosphoramidate (DEPA) and diisopropyl methylphosphonate (DIMP), methylphosphonic acid (MPA) hydrolysis products: ethyl methylphosphonate (EMPA), isopropyl methylphosphonate (IMPA), and pinacolyl methylphosphonate (PinMPA), and organophosphorus pesticides: dichlorvos and malathion were all purchased from Sigma Aldrich (St. Louis, MO, USA). V-series organosulfur hydrolysis products: 2-diethylaminoethanethiol (EDA) and 2-diisopropylaminoethanethiol (IDA) were purchased from AA Blocks (San Diego, CA, USA). The isotopically labelled internal standards d_7-IMPA and N-Acetyl-L-cysteine methyl ester (ACME) were purchased from Sigma Aldrich and

$^{13}Cd_3$ - DIMP internal standard was obtained from Dr. Bob Williams and Mark Alvarez at the Los Alamos National Laboratory (Los Alamos, NM, USA). Silver pSERS substrates were purchased from Metrohm USA, Inc. (Riverview, FL, USA). A 3 mm biopsy punch was purchased from Henry Schein Inc. (Melville, NY, USA). Whatman 31 ET chromatography paper was purchased from GE Healthcare Life Sciences (Pittsburg, PA, USA). The paper was laser cut using a Speedy 300 laser engraver from Trotec (Plymouth, MI, USA).

3.3.2 3D Printing SERS to PS Adapter

In order to combine paper-based SERS analysis to PS-MS, a part was designed to hold the SERS paper sample punch. The insert was designed with Sketchup and printed on an Ultimaker 2 Extended+ (Geldermalsen, Gelderland, Netherlands). Polypropylene filament with a diameter of 2.85 mm and nozzle diameter of 0.4 mm was used to print the design. Polypropylene tape was applied to the plate bed to assist with print adhesion. The nozzle temperature was 220 °C and the print bed was heated to 100 °C with a printing speed of 20 mm/s.

3.3.3 SERS Analysis

Raman spectra were collected on a B&W Tek iRaman Plus from Metrohm USA Inc. (Riverview, FL, USA). This instrument contains a 785 nm laser. A 3 mm punch was taken from the pSERS paper and placed into a 3D printed holder shown in Figure 3.2. A sample volume of 3 μL was drop cast onto the punch and was analyzed wet. Raman spectra were collected from the pSERS paper using 30% power and an average of 5 spectra at 10 seconds per scan. Dark subtraction and baseline corrected (Lambda = 1125) were performed on the spectra. Dilution series from 1 to 1000 part-per-million (ppm, mass to volume) for EDA, IDA, and malathion and from 3% to 50% for DIMP and DMMP) were analyzed to estimate the limit of detection for each analyte in the assay.

3.3.4 Paper Spray Analysis

The 3D printed holder containing the pSERS paper disc were loaded into the sample wells on a VeriSpray sample plate (Thermo Scientific, San Jose, CA) as shown in Figure 3.2B. Following addition of an internal standard solution, the sample plate was loaded into the plate

loader magazine for subsequent analysis by a VeriSpray automated paper spray source coupled to an Altis triple quadrupole mass spectrometer from Thermo Scientific (San Jose, CA, USA)[39-41]. Methanol with 0.1% formic acid was used as the spray solvent. The mass spectrometer was operated in dual polarity. The first 0.6 min of the method were in negative ion mode (NIM) and the second half of the method, 0.6-1.2 min, was in positive ion mode (PIM). The chemical warfare agent simulants (DEPA, DIMP, DMMP), V-series organosulfur hydrolysis products (EDA and IDA), and pesticides (dichlorvos and malathion) were analyzed in PIM. Four of the CWA methylphosphonic acid hydrolysis products (EMPA, IMPA, MPA, PinMPA) were analyzed in NIM[42, 43]. The voltages were 3.7 kV and 3 kV for PIM and NIM, respectively. The instrument parameters and internal standard for each analyte can be found in Table 3.1. While the sample plates utilized for the paper spray method could be viewed as consumables, they in fact can be reused by washing the plates and by replacing the paper substrate. These steps would eliminate waste production arising from the used sample plates.

To characterize paper spray MS performance, calibration solutions containing all of the analytes were prepared in water with concentrations ranging from 1.6 to 25 ppm (4.8 to 75 ng on paper). Standards (3 μL) and internal standard solution (3 μL) (5 ppm DIMP[C_{13}]-d_3 and ACME, 0.5 ppm IMPA-d_7) were spotted onto the pSERS punch and were analyzed within the sample insert. Tracefinder version 3.3 (Thermo Scientific) was used for mass spectral data analysis. Calibration curves were prepared by plotting the area under the curve (AUC) of the analyte versus AUC of the internal standard. The correlation coefficients were determined using 1/x weighted least squares regression line. The limits of detection were calculated by the standard error of the y-intercept, divided by the slope, and multiplied by a factor of 3. The relative slope error was calculated by dividing the standard error of the slope by the slope and multiplying by 100.

3.3.5 Sample Wipe

Two concentrations (333 ppm and 1000 ppm) of EDA, IDA, and malathion (10 μL) were spotted onto a surface and were allowed to dry. Methanol was used to wet the surface (5 μL) before wiping with a dry pSERS punch. The pSERS punch was rewet with water before SERS analysis (3 μL). Following SERS, the disk was placed in the insert for paper spray analysis.

Table 3.1 Analytes included in PS-MS method.

Analyte	Description	Polarity (+/-)	RF	CE (V)	Transition (m/z)	Internal Standard
DEPA	Simulant	+	45	14 10	154.1 → 98.1 154.1 → 126.0	DIMP[C_{13}]-d_3
Dichlorvos	Pesticide	+	55 57	21 25	220.9 → 109.0 220.9 → 126.9	DIMP[C_{13}]-d_3
DIMP	Simulant	+	54	14	181.1 → 97.0	DIMP[C_{13}]-d_3
DMMP	Simulant	+	46	24	125.0 → 62.9	DIMP[C_{13}]-d_3
EDA	Hydrolysis Product	+	30	16	133.2 → 86.2	ACME
EMPA	Hydrolysis Product	-	30	46 14	123.0 → 63.0 123.0 → 95.1	IMPA-d_7
IDA	Hydrolysis Product	+	30	16 18	161.4 → 118.1 161.4 → 86.1	ACME
IMPA	Hydrolysis Product	-	41	27 15	137.0 → 77.1 137.0 → 95.1	IMPA-d_7
Malathion	Pesticide	+	54 60	13 23	331.0 → 127.0 331.0 → 99.0	DIMP[C_{13}]-d_3
MPA	Hydrolysis Product	-	46	18 45	95.0 → 79.0 95.0 → 63.0	IMPA-d_7
PinMPA	Hydrolysis Product	-	46	55 17	179.1 → 63.1 179.1 → 95.1	IMPA-d_7
ACME	IS	+	30	11	178.1 → 135.9	
DIMP[C_{13}]-d_3	IS	+	35	14	185.1 → 101.0	
IMPA-d_7	IS	-	41	15	144.0 → 96.0	

The table includes a description of the compound, the collision energy, the RF values, the polarity used for analysis, the selected reaction monitoring (SRM) transitions, and the deuterated standard utilized for each analyte.

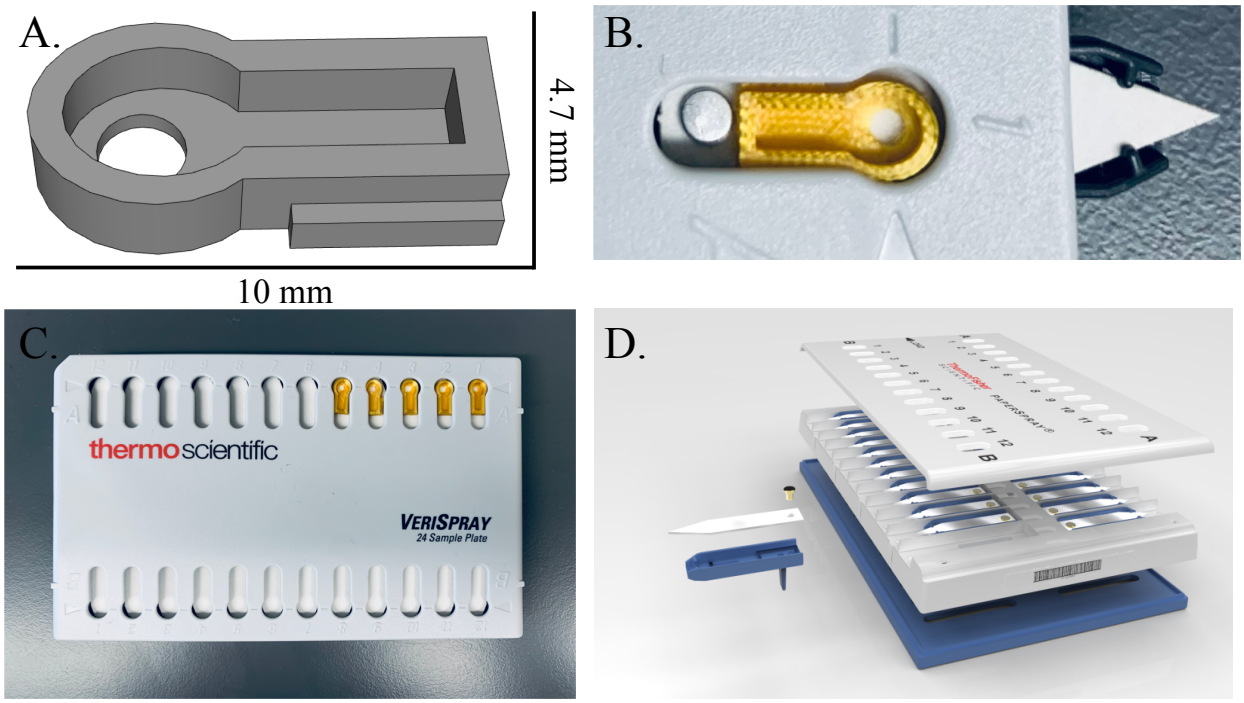

Figure 3.2 (A) Rendering of 3D printed cartridge insert for sample analysis. (B) Close-up of pSERS insert with paper spray tip extended for analysis. (C) Image of pSERS inserts (gold) in the paper spray sample plate. (D) Cross section of VeriSpray plate assembly.

3.4 Results and Discussion

3.4.1 Workflow

Figure 3.1 shows the overall workflow from wipe collection, to fieldable screening by Raman, to confirmation by mass spectrometry. A 3D printed piece was designed to facilitate the transition between SERS and PS (Figure 3.2). Figure 3.2A shows a rendering of the piece while Figure 3.2B shows the printed insert contained within the paper spray plate for MS analysis. The 3D printed insert holds the 3 mm sample punch and has a small hole where analyte, dissolved in solvent, can wick through to the Whatman 31 ET paper spray tip underneath.

3.4.2 SERS

Following sample collection, samples were first analyzed with SERS. Characteristic spectra were obtained for five analytes in the assay, shown in Figure 3.3. A range of concentrations were drop cast onto the SERS punch. For EDA, IDA, and malathion the concentrations ranged

from 1 to 1000 ppm and 3 to 50% for DIMP and DMMP. EDA and IDA, which are structurally similar, showed strong peaks at 641 cm^{-1} and 718 cm^{-1} corresponding to the C-S stretch[44, 45]. The malathion SERS spectrum differed significantly from the ethanethiol molecules. It has strong a strong peak at 510 cm^{-1} for the P-S stretch and 636 cm^{-1} for the P=S stretch[46]. DIMP and DMMP both share a characteristic stretch at 710 cm^{-1} indicating a P-C stretch[47].

For some compounds, as the concentration increases, the signal intensity plateaus. This is not uncommon in SERS analysis and calibration curves often follow the Langmuir adsorption model[48-50]. Although not the focus of this study, a calibration curve could be fitted to the points for a pseudo-quantitative model. As seen in Fig. 3A and B, the intensities for 3000 ng of EDA and IDA are lower than 300 ng. This is due to the SERS surface being saturated with analyte molecules and exceeding monolayer coverage which in turn causes the signal to decrease[51]. DIMP and DMMP could only be detected at concentrations of ~3% and higher, compared to the ppm level detection for than EDA, IDA or malathion. To determine if SERS enhancement was taking place despite the poor detection limits, a 50% concentration was spotted onto regular paper and compared to the pSERS paper. While signals corresponding to DIMP and DMMP were obtained from plain paper, the signal intensities were 2x and 6x higher for DIMP and DMMP respectively when using the pSERS paper. No peaks were detected for EDA, IDA, or malathion when analyzed from plain paper at 3000 ng, indicating the importance of SERS for detecting ng concentrations of these analytes by Raman.

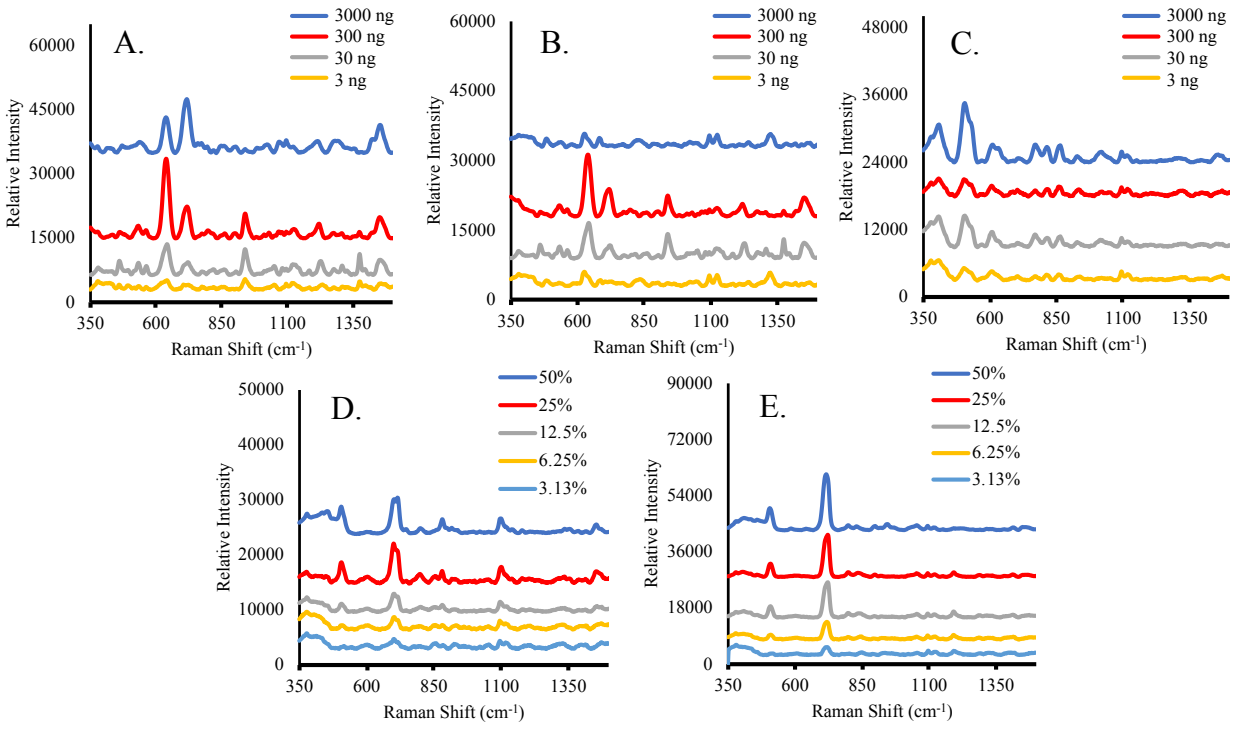

Figure 3.3 SERS spectra of EDA (A), IDA (B), malathion (C), DMMP (D), DIMP (E)

The alkyl methylphosphonic acid (MPA) hydrolysis products (Table 3.1) are often difficult to detect with SERS[52-54]. These organophosphonic acids and the DMMP and DIMP analytes have a weak interaction with the noble metal nanoparticle substrate[52]. Therefore, there is little to no enhancement of the Raman signal via SERS[53, 54]. EMPA and IMPA were detected at 50% concentrations, however, the SERS paper did not provide any enhancement of the signal compared to bulk Raman. Surprisingly, PinMPA was detected down to 2000 ppm using SERS paper, much lower than the other organophosphonic acids (Figure 3.4). PinMPA was not detected at any concentration using bulk Raman without surface enhancement. Further work could be done to create a substrate capable of capturing the acidic hydrolysis products and pulling them closer to the nanoparticle surface. Zhao et al., for example, employed this approach by using a linker molecule (2-aminoethanethiol) to induce an amidation reaction with the MPAs[53].

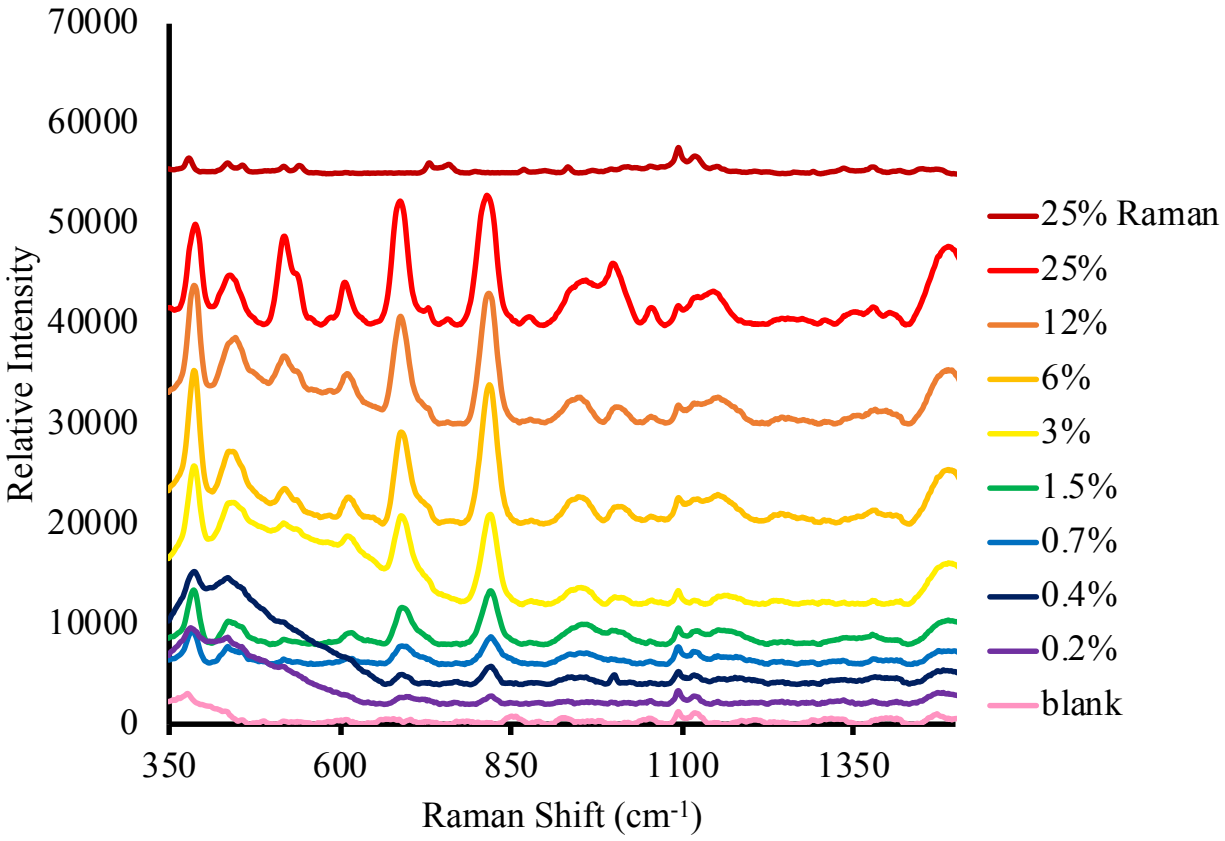

Figure 3.4 . PinMPA SERS dilution series spectra

3.4.3 PS-MS

After analysis by SERS, the paper was placed into the 3D printed insert shown in Figure 2. This insert enabled automated paper spray analysis on a triple quadrupole mass spectrometer. SRM ratios for obtained for four of the analytes are shown in Figure 3.5, one of each sub-type of organophosphate is represented in the figure: EMPA (methylphosphonic acid hydrolysis product), DEPA (CWA simulant), EDA (V-series organosulfur hydrolysis product), and malathion (pesticide). Calibration curves using pSERS paper punches and the 3D printed inserts were created (Figure 3.6). The limits of detection, correlation coefficients and relative slope errors for the assay are detailed in Table 3.2. The limits of detection are in the low ng range with many at or below that level. Correlation coefficients ranging from 0.94 to 0.99 shows the potential for semi-quantitation. The relative slope error was below 10% indicating that the points fall near the regression line. Due to the non-ideal internal standards, EDA and IDA showed higher variance and poorer linearity. While an internal standard that more closely matches the chemical and

physical properties of those molecules would improve quantitation, the current performance is adequate considering our focus on fast, relatively simple screening applications.

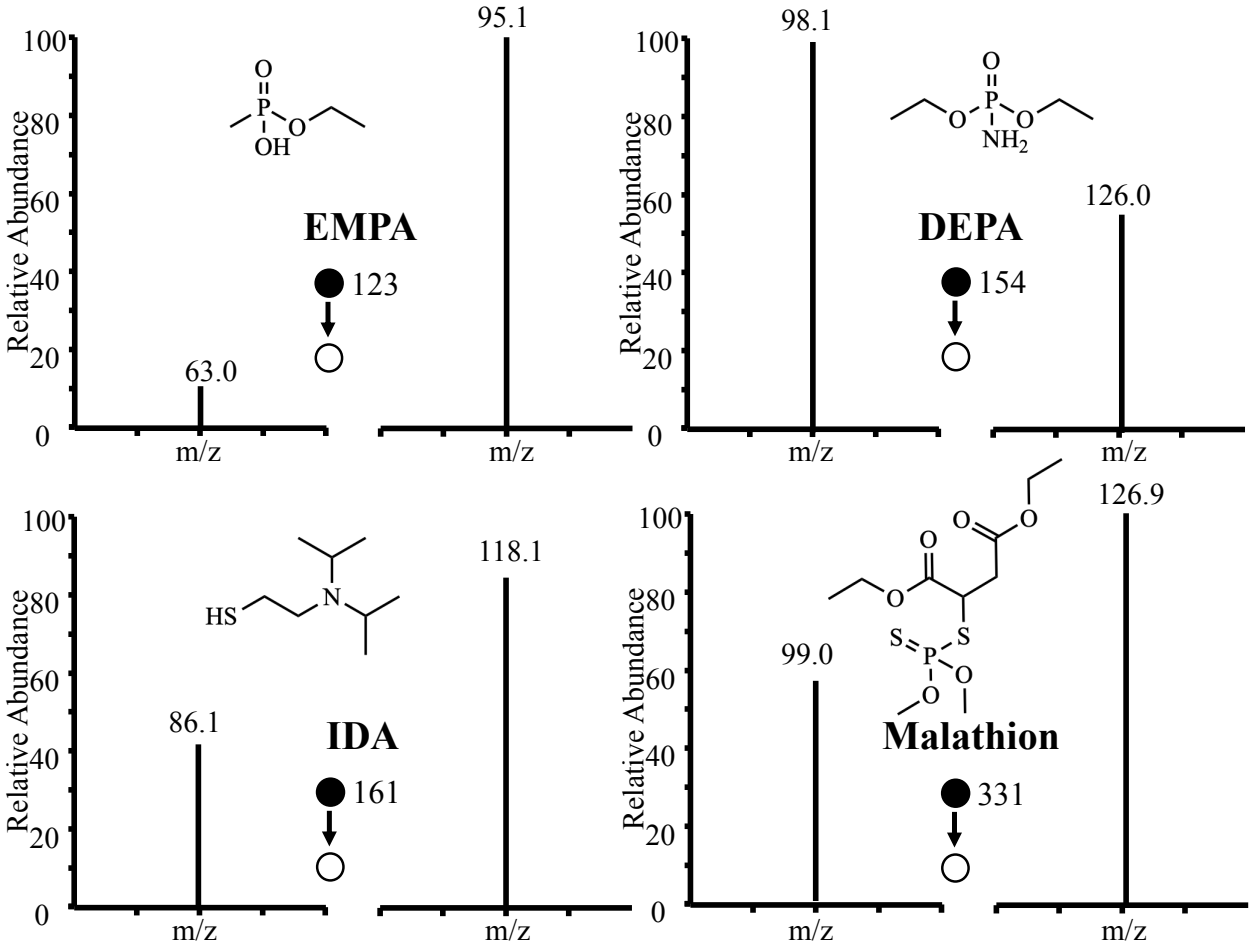

Figure 3.5 SRM ratio mass spectra for EMPA, DEPA, IDA and malathion

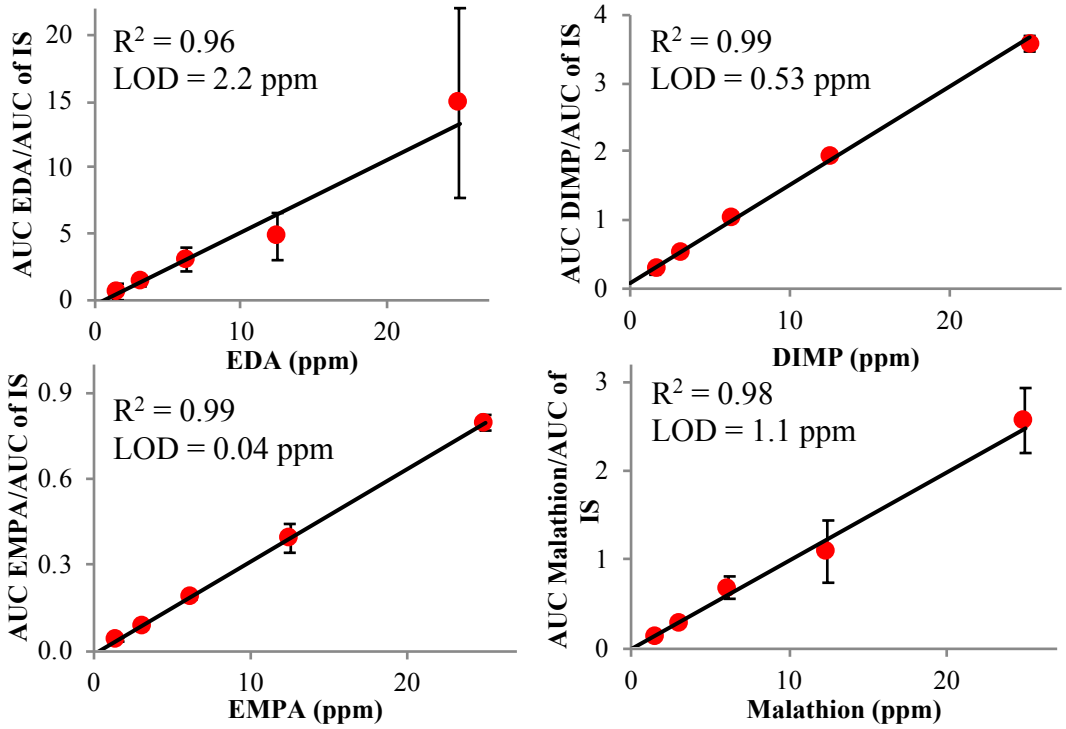

Figure 3.6. Select Paper spray mass spectrometry calibration curves

Table 3.2 Correlation coefficients, limits of detection, and relative errors of the slope for compounds in PS-MS assay.

	LOD (ng)	R^2	Relative Slope Error (%)
DEPA $[M+H]^+$	2.4	0.99	4.1
Dichlorvos $[M+H]^+$	0.6	0.99	1.2
DIMP $[M+H]^+$	1.5	0.99	2.8
DMMP $[M+H]^+$	3.0	0.99	5.1
EDA $[M+H]^+$	6.6	0.96	11.5
EMPA $[M-H]^-$	0.12	0.99	0.2
IDA $[M+H]^+$	7.8	0.94	13.9
IMPA $[M-H]^-$	0.6	0.99	1.2
Malathion $[M+H]^+$	3.3	0.98	5.9
MPA $[M-H]^-$	0.12	0.99	0.2
PinMPA $[M-H]^-$	1.2	0.99	2.3

3.4.4 Sample Wipe

Malathion, EDA and IDA were wiped off a tabletop surface to explore detection of residues from surfaces. The overlayed blank and sample SERS spectra and chronograms are shown in Figure 3.7 for three analytes. The SERS signal intensity is lower than would be expected for the same concentration spotted directly onto the pSERS paper. This could be due to dilution during the wiping protocol or incomplete recovery when wiping the sample off the surface. However, the overlayed chronograms for the wiped samples versus the blank shows that the compounds were detected from the wipe (Figure 3.7). This proof of concept experiment shows potential to recover samples off surfaces via wiping at microgram levels for subsequent screening and confirmation via SERS-MS. A wider variety of surfaces will need to be tested and the wiping protocol will need to be improved before it can be used in the field.

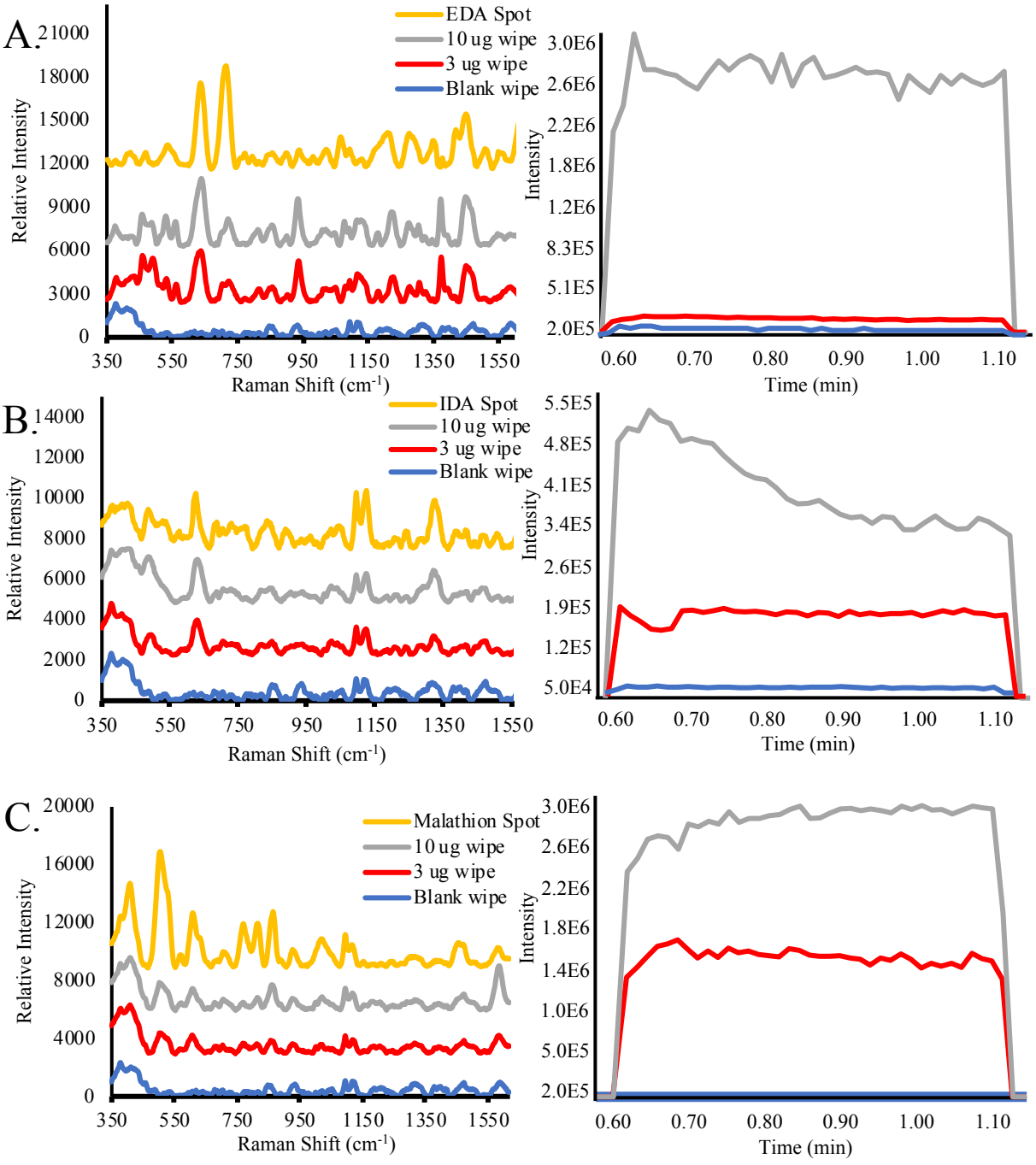

Figure 3.7 SERS spectra and overlayed chronograms for (A) EDA, (B) IDA, and (C) malathion after wiping 3 and 10 μg off of a surface

3.5 Conclusion

In this study, a combined SERS-MS approach was developed to combine nondestructive screening using by Raman spectroscopy with subsequent confirmation by mass spectrometry. A device was developed and 3D printed to facilitate both analytical methods from the sample paper disc. After adding a sample to a silver pSERS paper substrate, it was analyzed first using Raman spectroscopy and then directly analyzed using PS-MS using an automated PS source coupled to a triple quadrupole mass spectrometer. A dual-polarity mass spectrometry method was utilized to analyze CWA simulants, CWA hydrolysis/degradation products, and organophosphorus pesticides. Finally, a proof-of-concept wiping experiment was performed to show the potential for field analysis of dried residues. The SERS assay detailed in this work can be performed in the field, for rapid screening of organophosphorus chemicals, which can then be followed by lab-based confirmation using the PS-MS assay. Future work will focus on wiping chemicals off a variety of surfaces and a specialized pSERS substrate will be developed for the analysis of the MPAs. Additionally, realistic field samples, such as mixtures, should be analyzed to determine detectability in the presence of interferences. Nevertheless, the coupling of these green analytical techniques has the potential to provide rapid pre-screening of a sample in the field, for the detection of harmful organophosphorus chemicals.

3.6 Acknowledgements

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CHAPTER 4. PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS) SCREENING IN WHOLE BLOOD USING PAPER SPRAY MASS SPECTROMETRY

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4.1 Abstract

Per- and polyfluoroalkyl substances (PFAS) are a group of environmental pollutants that have been highlighted in recent years due to their toxicity and propensity to bioaccumulate. In this work, paper spray mass spectrometry (PS-MS) was utilized to screen for the presence per- and polyfluoroalkyl substances in whole blood without sample pretreatment or cleanup. Eleven PFAS compounds were included in the targeted screening method. Single donor whole blood was spiked with varying concentrations of PFAS, and 5 μL of this spiked blood was spotted onto a paper substrate and allowed to dry. The substrate was positioned in front of a quadrupole-orbitrap mass spectrometer that was operated in negative ion mode using targeted MS/MS fragmentation. To improve the assay's performance, solvent and spot size optimization experiments were completed. Limits of detection ranged from 1.4-9.2 ppb with the optimized method.

4.2 Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of persistent organic pollutants of concern due to their toxicity and ability to bioaccumulate in humans and animals[1]. High levels of PFAS exposure have been linked to a wide array of adverse health effects, including decreased immune response, increased cholesterol levels, and increased risk for some cancers[2]. PFAS contain many carbon-fluorine bonds, which are the strongest single bonds in organic chemistry (130 kcal/mol)[3-5]. Due to this high bond strength, PFAS are often known as "forever chemicals" and are resistant to break down in the environment[6]. Since their widespread commercialization in the 1940s, PFAS have been utilized for many consumer products and industrial applications[7]. Many

nonstick or water repellant products, such as Polytetrafluoroethylene (PTFE)-coated cookware and upholstery protector sprays, contain PFAS molecules. Additionally, PFAS can be found in lifesaving products such aqueous film forming foams (AFFF), which are used to suppress hydrocarbon fires[8]. Currently there are approximately 14,000 registered PFAS molecules[9], and nearly all individuals have some level of PFAS in their blood[10, 11].

Although most of the population has some exposure to PFAS through commercial goods, individuals that have to regularly use AFFF to contain petroleum fires have significantly higher risk of exposure. Recent environmental studies have shown that the areas around military bases and airports, where AFFF are used, have increased PFAS contamination of ground water[7, 12, 13]. In June 2022, the EPA reduced its previous lifetime health advisory of 70 part-per-trillion (PFOA and PFOS) to less than 1 ppt (PFOA and PFOS) in water[14, 15]. Unfortunately, some military AFFF training sites have groundwater levels in the part-per-billion (ppb) range, making the levels approximately a million times higher than the EPA recommended concentration[12]. Military personnel and other individuals who work with and around AFFF have increased levels of PFAS in their blood due to increased exposure via inhalation of AFFF fumes and absorption of the chemicals through their skin[16, 17]. Recent studies by researchers who compared PFAS levels in firefighters within multiple fire departments in the United States highlight the elevation of PFAS in firefighters when compared to the general population[17]. A similar study in Australian firefighters had detected various PFAS compounds at median ranges between 1.5 ng/ml and 14 ng/ml, with some PFAS compounds in concentrations exceeding five times the average population[16].

The current gold standard for PFAS analysis in biofluids is through liquid chromatography and tandem mass spectrometry[18]. Additionally, Baker and coworkers have shown the applicability of liquid chromatography-ion mobility-mass spectrometry for the untargeted identification of PFAS[19]. While sensitive, methods utilizing liquid chromatographic separations are costly and time consuming, limiting widespread screening of highly exposed populations. Ambient ionization techniques, that require far less sample clean-up, would be aptly suited for this type of analysis. Fedick and coworkers developed a method to detect PFAS in raw soil samples, without extraction, using cone spray coupled directly to mass spectrometry[20]. Additionally, Seró, et al. developed a method to detect PFAS in waterproofing products using paper spray coupled to atmospheric pressure photoionization[21]. Paper spray mass spectrometry (PS-MS) is an ambient ionization technique that was first developed in the 2010s as a way to detect small molecules in dried blood

spots without extraction[22, 23]. Since its development there have been a multitude of studies on the advancement of the technique and showcasing its use in a wide variety of fields and as a solution to diverse analytical problems[21, 24-36].

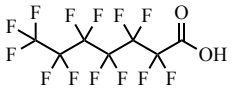
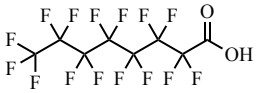
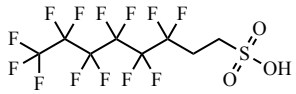
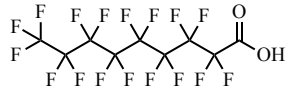
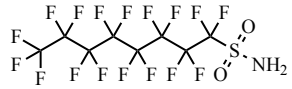
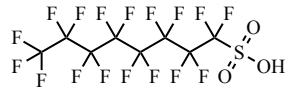
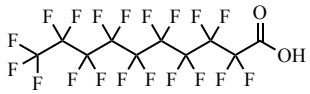
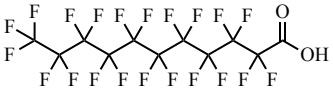
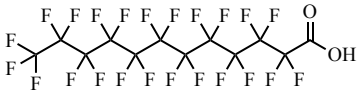
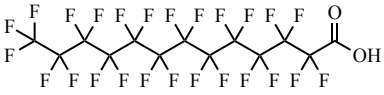
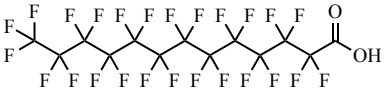
In this work, paper spray mass spectrometry (PS-MS) was utilized for detecting PFAS in whole blood. This method could provide an alternative approach for screening at-risk groups for harmful PFAS chemicals in the blood. Earlier detection would provide individuals with more options regarding medical monitoring and would hopefully reduce the overall risk of developing adverse health effects such as cancer.

4.3 Experimental Methods

4.3.1 Chemicals and Materials

Solid PFAS standards (Table 4.1) were obtained from Dr. Patrick Fedick at the Naval Air Warfare Center Weapons Division. Internal standard (IS) solution of PFOS, PFOA, PFHxS (^{13}C, 99%) at 2000 ng/mL was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC grade water, methanol, acetonitrile, formic acid, ammonium hydroxide, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), carbon tetrachloride (CCl_4), ammonium hydroxide, and Whatman 31 ET chromatography paper were purchased from Fisher Scientific (Hampton, NH, USA). Paper spray cartridges were purchased from Prosolia Inc. (Indianapolis, IN, USA).

Table 4.1 PFAS standards utilized in assay development

PFAS	Structure	MW (g/mol)	$[M-H]^-$ (m/z)	CE	MS/MS quan fragment (m/z)	Internal Standard
Perfluoroheptanoic acid (PFHpA)		364.1	362.9	25	118.9922	PFOA-$^{13}C_8$
Perfluorooctanoic acid (PFOA)		414.1	412.9	20	368.9753	PFOA-$^{13}C_8$
Fluorotelomer sulfonic acid 6:2 (6:2 FtS)		428.2	426.9	30	406.9605	PFOA-$^{13}C_8$
Perfluorononanoic acid (PFNA)		464.1	462.9	15	418.9723	PFOA-$^{13}C_8$
Perfluorooctane-sulfonamide (PFOSA)		499.2	497.9	25	77.9652	PFOS-$^{13}C_8$
Perfluorooctanesulfonic acid (PFOS)		500.1	498.9	45	168.9887	PFOS-$^{13}C_8$
Perfluorodecanoic acid (PFDA)		514.1	512.9	18	268.9820	PFOA-$^{13}C_8$
Perfluoroundecanoic acid (PFUdA)		564.1	562.9	15	518.9656	PFOA-$^{13}C_8$
Perfluorododecanoic acid (PFDoA)		614.1	612.9	13	568.9623	PFOA-$^{13}C_8$
Perfluorotridecanoic acid (PFTrDA)		664.1	663.0	12	618.9591	PFOS-$^{13}C_8$
Perfluorotetradecanoic acid (PFTeDA)		713.0	662.9	15	668.9561	PFOA-$^{13}C_8$

4.3.2 Sample Preparation

Solid PFAS standards were dissolved in 96:4 methanol:water at a 1000 part-per-million (ppm, mass:volume) concentration[37]. All samples were stored in polypropylene vials as noted in EPA Method 537.1 to prevent adsorption. All standards were combined and diluted to 6 ppm in 96:4 methanol:water. From there, the solution was diluted to 2.4, 0.96, 0.38, 0.15 ppm in 96:4 methanol:water. These spiking solutions were used to spike into blood and create calibration curves.

Single donor whole blood was used for analysis. To create calibrators, whole blood (195 μL) was spike with 5 μL of calibration solutions (0.15-6 ppm). The resulting concentrations in blood ranged from 3.8 to 150 part-per-billion (ppb, mass:volume). Isotopically labeled internal standards (IS) were also added to the blood samples (2.5 μL of 2000 ppb IS).

4.3.3 Paper Spray

Teardrop-shaped paper tips were laser cut using a VLS 2.30 laser engraver with a CO_2 laser from Universal Laser Systems (Scottsdale, AZ). Spiked blood samples (5-10 μL) were spotted into the center of the paper and were allowed to dry for approximately 30 minutes prior to analysis. Following the dry time, samples were loaded into a holder built in-house to facilitate paper spray (Figure 4.1). The holder has an X, Y, Z stage that slides onto a mount connected to the mass spectrometer. A high voltage cable runs from the high voltage port on the MS to a copper connector that contacts the ball bearing inside the paper spray cartridge (Figure 4.1). The paper spray tip was positioned approximately 5 mm away from the ion transfer tube of the mass spectrometer. An extraction/spray solvent (150-200 μL) was applied to the cartridge in the rear well. The method was not started until the solvent had visually wicked through the paper and dried blood spot to the tip (approximately 5 seconds). After application of 3000 V in negative ion mode, an electrospray-like event occurs at the tip of the paper. The voltage was set to 0 V for the first and last 0.1 minutes of the 1-minute method to bring the signal down to the baseline to facilitate automatic peak integration.

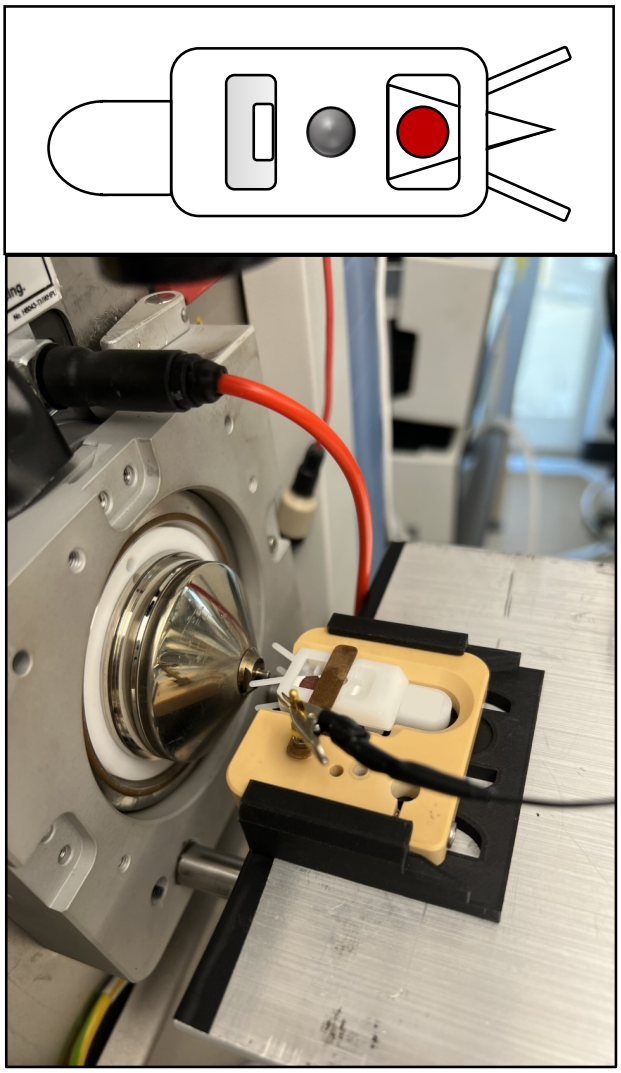


Figure 4.1 Manual paper spray set-up (bottom); paper spray cartridge (top)

To improve the performance of the assay, spray solvent and sample spot size were optimized. Six solvents/solvent blends were utilized in the optimization: 90:10 methanol:carbon tetrachloride with 0.1% ammonium hydroxide (v:v), 90:10 methanol:carbon tetrachloride with 0.1% formic acid, 90:10 methanol:trifluoroethanol, methanol with 0.05% CHAPS, 90:10 acetonitrile:carbon tetrachloride with 0.1% ammonium hydroxide, 90:10 methanol:water with 0.1% ammonium hydroxide. Three solvent spot sizes: 5, 7.5, and 10 µL were compared.

4.3.4 Mass Spectrometry and Data Analysis

For this study, a Q-Exactive Focus Mass Spectrometer from Thermo Scientific (San Jose, CA, USA) was utilized. The ion transfer tube was held at 320 °C, the S-lens was set to 50, and the resolution was 35,000. The system was operated in parallel reaction monitoring (PRM) (MS/MS mode using an inclusion list) with an isolation width of ±0.5 m/z. The fragments and collision energies were optimized for each compound by ramping the collision energy from 0-100 (Table 1). PRM fragmentation provides not just fragmentation information for one specific fragment, but all fragment ions arising from the precursor ion. Therefore, ions not used for quantitation, can be used for as qualifier ions for true unknown samples. The instrument was calibrated once every seven days, and the ion transfer tube was changed each day before analysis.

Data analysis was performed using Xcalibur Qual Tracefinder Version 3.3 (Thermo Scientific). Tracefinder was used to automatically integrate chronograms with a 5 ppm mass precision. Calibration curves were fit with a 1/x weighted least squares regression. The limits of detection were determined by the ratio of the standard error and the y-intercept of the slope, multiplied by 3. In order to identify a true unknown sample as being positive for a specific PFAS molecule, the detected concentration would need to be above the limit of detection and contain one or more qualifier ions. The area under the curve (AUC) and signal-to-blank (S:B) was used for comparing different optimizations. Due to the high resolution and accurate mass of this instrument, sometimes the blank samples (whole blood without spiked in PFAS), did not have integratable signal. In those cases, an approximation was made using Xcalibur Qual Browser. Qual Browser can be used to find the instrument noise of a measurement; however, it cannot be used for comparison with an AUC since the noise noted on the spectra is for a single scan. The following relationship was used for approximating the AUC of the noise:

$$\frac{Noise\ Intensity * Analyte\ AUC}{Analyte\ Intensity} = Noise\ AUC$$

4.4 Results and Discussion

4.4.1 Paper Spray of PFAS Molecules

Blood samples were spotted onto a paper spray cartridge and were allowed to dry. The extraction/spray solvent was manually applied to the rear of the cartridge. The solvent wicked through both the paper and the blood spot as it traveled from the rear to the front of the paper spray cartridge. As the solvent wicked through the blood spot, it extracted the PFAS molecules. Following the potential application, an electrospray plume occurred at the tip of the paper, ionizing the molecules of interest. After application of the spray voltage, the ions are detected immediately by the mass spectrometer. Since there is no chromatographic separation prior to MS analysis, the resulting graph of signal intensity over time is known as a chronogram (Figure 4.2). The method was designed to apply no voltage for the first and final 0.1 minutes of the method and 3 kV for the middle 0.8 minutes of the method. This was done to ensure that the data processing software would fully integrate the area under the curve (AUC) for the analytical run.

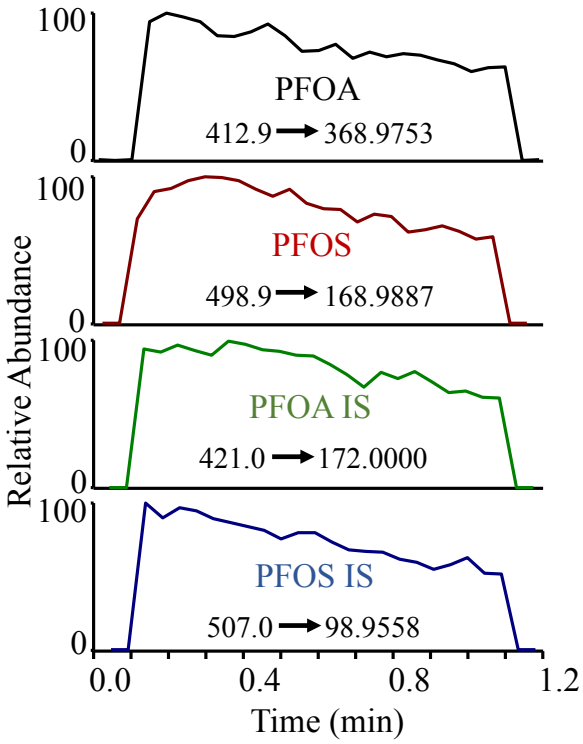


Figure 4.2 Extracted ion chronograms for PFOA, PFOS, PFOA-$^{13}C_8$, PFOS-$^{13}C_8$ in whole blood

Due to the complex nature of blood matrix, PFAS are oftentimes at a much lower concentration than other ionizable constituents in the sample. The PFAS precursor and fragment ions are not the dominating peaks in the mass spectra. However, sensitivity and specificity can still be accomplished using MS/MS fragmentation coupled to the high mass accuracy of the Orbitrap instrument. As PFAS molecules are fragmented in the HCD cell of the instrument, they break into reproducible fragment ions that can be detected simultaneously using parallel reaction monitoring. The presence of more than one fragment ion increases the specificity for confirmation of the presence of the target PFAS molecule.

4.4.2 Solvent Optimization

Previous works have shown that the spray solvent selected for a paper spray assay has a significant effect on the analytical performance. Recent work by Skaggs et. al. show how spray solvent optimization can increase the signal-to-blank by up to three orders of magnitude [34]. Additionally, paper spray assay development in negative ion mode has often lagged behind its positive counterpart, due to the frequency and ease of corona discharge. In this work, the spray solvent was optimized to stabilize the Taylor cone and reduce the propensity for electrical discharge. A variety of solvent blends (Table 4.2) were selected that have been shown in the literature to suppress discharge in negative ion mode electrospray ionization or are typical paper spray solvents included for comparison to the more niche blends. Three blank replicates and three test replicates were performed for each solvent type. Figure 4.3 shows the average signal divided by the average blank signal the solvent study.

Table 4.2 Solvents utilized in the solvent study and the reasons they were chosen

Solvent Number	Solvent Blend (v/v)	Reasoning
Solvent 1	90/10 Methanol/Carbon tetrachloride with 0.1% Ammonium hydroxide	Negative ion mode solvent previously reported to reduce discharge in negative ion mode [38]
Solvent 2	90/10 Methanol/Carbon tetrachloride with 0.1% Formic acid	Assess addition of weak acid in negative ion mode [39]
Solvent 3	90/10 Methanol/Trifluoroethanol	Trifluoroethanol shown to suppress discharge [40]
Solvent 4	Methanol with 0.05% CHAPS	CHAPS improves spray stability [41]
Solvent 5	90/10 Acetonitrile/Carbon tetrachloride with 0.1% Ammonium hydroxide	Polar aprotic solvent
Solvent 6	90/10 Methanol/Water with 0.1% Ammonium hydroxide	Inclusion of water to mimic a typical paper spray solvent [42]

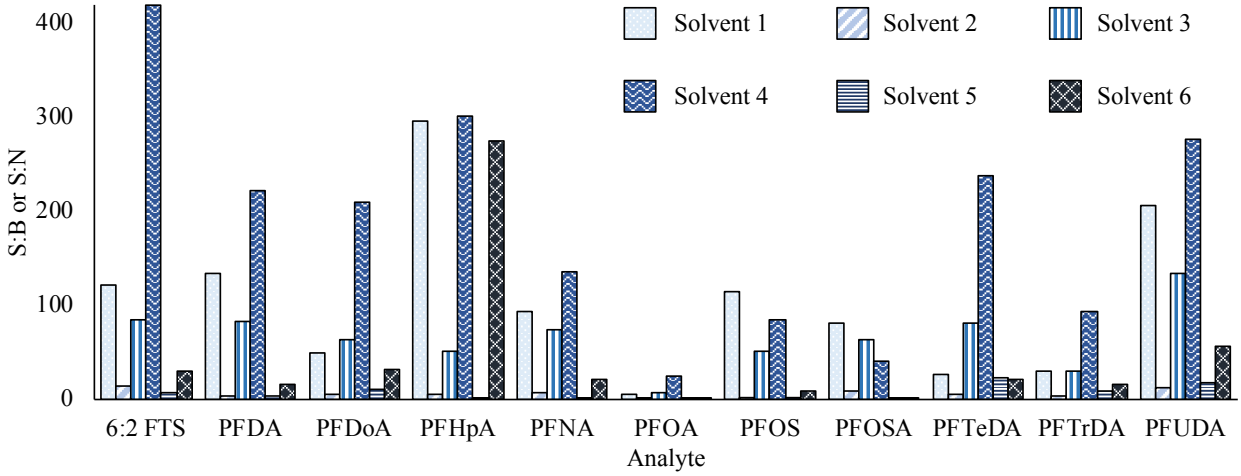


Figure 4.3 Results from the solvent study for the quan ions for each analyte reported in Table 1. Due to the increase in S:B/S:N for most of the analytes, solvent 4 (methanol with 0.05% CHAPS) was selected for future experiments and method development

Solvent 4 (methanol with 0.05% CHAPS) yielded the best the S:B or S:N for the majority of the analytes (9 out of 11). While some analytes like PFHpA had similar results with multiple solvent types, for others like PFOA or 6:2 FTS the solvent significantly improved the signal relative to the other blends tested. Additionally, the total ion chronogram (TIC) for solvent 4 was stable for all of the blanks and test replicates (6/6 samples). A sample was labeled as having a

stable TIC if the signal intensity was mostly consistent over the 1-minute analysis period (Figure 4.4). Solvent 1 was previously used to reduce discharge and was the best performing after solvent 4, in terms of the signal to blank value. However, only 2/6 samples had a stable TIC. Solvent 2 was identical to solvent 1 with the addition of formic acid instead of ammonium hydroxide. The formic acid solvent was the worst performing across the analyte panel in terms of the S:B, but had 4/6 stable TICs. The 90/10 Methanol/trifluoroethanol solvent system performed moderately (5/6 stable TICs), but not as well as solvent 4. Acetonitrile (solvent 5) did not wick through the bloodspot, resulting in poor S:B values and 4/6 stable TICs. The methanol/water mixture of solvent 6 did not perform well for any PFAS except PFHpA, which is unsurprising due to the ease of discharge in negative ion mode when water is present[43]. Additionally, only half of the samples had a stable total ion chronogram for the water-containing solvent.

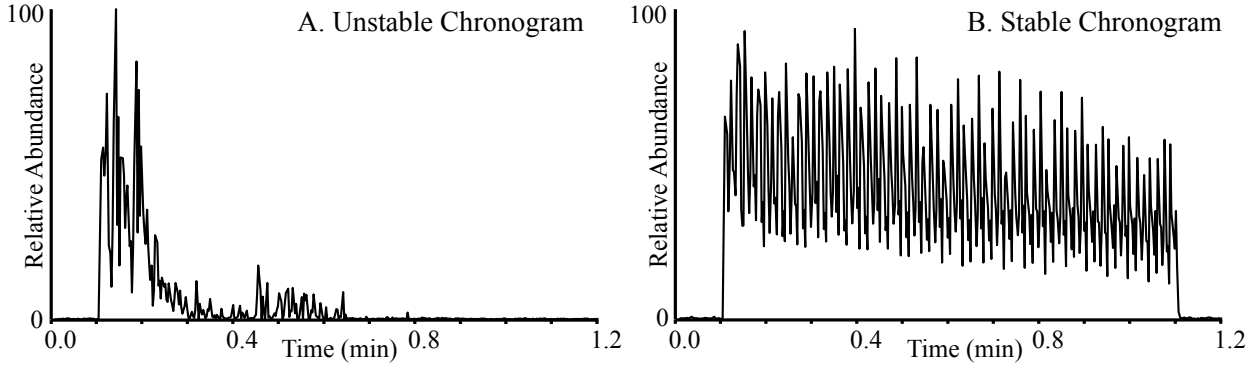


Figure 4.4 Examples of an (A) unstable and (B) stable total ion chronogram. Solvent 1 and solvent 4 were used for the unstable and stable chronogram, respectively

4.4.3 Spot Size

Although the spray solvent was optimized for PFAS molecule sensitivity, the stability was still an unaddressed issue. It was observed that the formation of a stable Taylor cone was inconsistent, and sometimes the sample would not spray at all. It was hypothesized that the 10 μL sample spot used in the initial method development experiments was creating a barrier that the solvent could not wick through effectively. This hypothesis was tested by using different sized sample spots. In this work 5, 7.5, and 10 μL blood sample spots were evaluated for their spray stability. The 10 μL spot spanned the width of the paper, whereas the 5 and 7.5 μL samples did not. Blood samples were spiked with PFAS to a 24 ppb final concentration and were spotted onto

the spray substrate before analysis. Three replicates were analyzed and were averaged for the signal-to-blank calculations. The percent relative standard deviations (% RSD) was then calculated on the three replicate AUC signals to assess reproducibility between replicate analyses. It was found that the smallest sample spot had the most reproducible analysis, and oftentimes the highest signal-to-blank. The average % RSD for 5, 7.5, and 10 μL was 34, 60 and 59, indicating that the replicates for 5 μL were more reproducible than those at 7.5 and 10 μL. While most of the analytes also had the highest S:B at the 5 μL level, PFDoA and PFOSA, had highest S:B ratios for 7.5 μL and 10 μL, respectively. Across the analyte panel, the 5 μL spot size had the lowest %RSD.

4.4.4 Analytical Performance

Following the method optimizations, calibration curves were created using spiked single donor blood to assess the sensitivity and reproducibility of the assay. In the final method, 5 μL of calibrator samples were spotted onto laser cut paper substrates and were allowed to dry for 30 minutes. Each sample was analyzed manually by applying 200 μL of methanol with 0.05% CHAPS as the spray solvent. After the application of the voltage, the Taylor cone formation was monitored using a camera. Due to the optimizations, there were no discharge sparks near the tip of the paper substrate. The chronograms were consistent for the duration of the analysis. Calibration curves were constructed by taking the area under the curve of the quantitative fragment ions relative to the area under the curve of the internal standard. Figure 4.5 shows the select calibration curves for three analytes PFOA, PFUDA and 6:2 FTS. Each point is an average of three replicates. The LODs and correlation coefficients are listed in Table 3. The calibration curve correlation coefficients ranged from 0.83-0.99, indicating the potential for semi-quantitation. The limits of detection were in the low ppb range, ranging from 1.4-9.2 ppb (Table 4.3). As shown in Figure 4.5, there is some variability in the replicate measurements that would hinder the quantitative capabilities of the method. The use of isotopically labeled internal standards is critical for the quantitative performance. Without an internal standard, the limits of detection are approximately three times higher and the correlation coefficients decrease to 0.75-0.93. Because only two internal standard molecules were used in this study, there is a potential to improve the quantitation by expanding the internal standards utilized. The limits of detection reported here were adequate for detecting concentrations of PFAS in blood for individuals with high levels of occupational

exposure to PFAS or AFFF[44, 45]. For example, Leary, et al. investigated the association between PFAS occupational exposure and metabolic syndrome detected blood serum concentration levels in firefighters ranging from 1.57 to 30.42 ng/mL for PFOS, 1.02-4.65 ng/mL for PFOA, 0.84-22.49 ng/mL for PFHxS, and 0.2-1.36 ng/mL for PFNA[45].

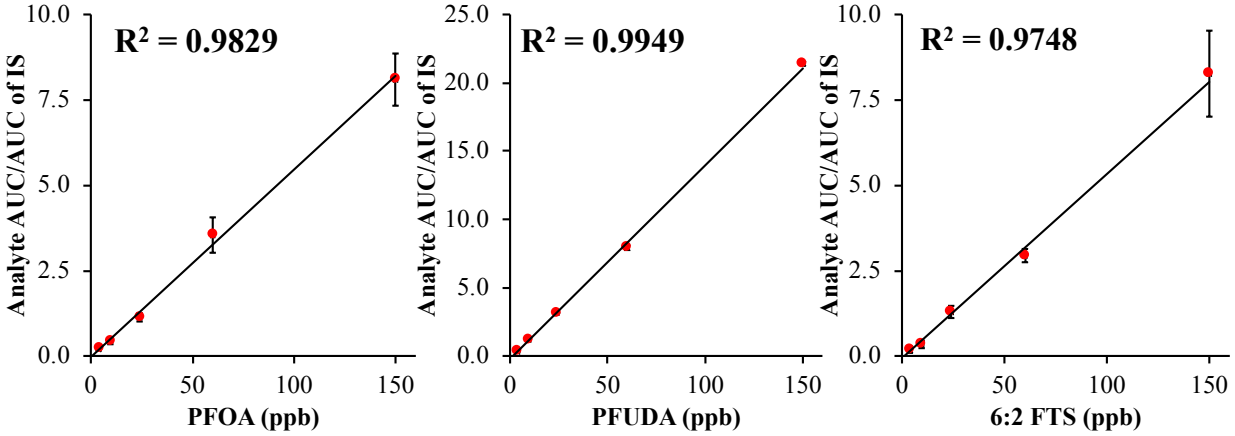


Figure 4.5 Select calibration curves for PFAS detection in whole blood using PS-MS without protein precipitation. Each point is an average of three replicates

Table 4.3 Analytical performance of PS-MS method

	Quan Peak	IS	R^2	LOD (ppb)
6:2 FTS	406.9605	PFOA IS	0.9748	3.2
PFDA	268.982	PFOA IS	0.9840	2.6
PFDoA	568.9623	PFOA IS	0.9935	1.6
PFHpA	118.9922	PFOA IS	0.9704	3.5
PFNA	418.9723	PFOA IS	0.9949	1.4
PFOA	368.9753	PFOA IS	0.9829	2.6
PFOS	168.9887	PFOS IS	0.9346	5.3
PFOSA	77.9652	PFOS IS	0.8265	9.2
PFTeDA	668.9561	PFOA IS	0.9667	3.7
PFTrDA	618.9591	PFOS IS	0.9830	2.6
PFUDA	518.9656	PFOA IS	0.9900	2.0

4.5 Conclusion

In this work, a method was developed to rapidly screen whole blood samples for PFAS exposure. While many PFAS biofluid assays utilize LC-MS/MS, this work focused on a rapid, simplified approach by means of paper spray mass spectrometry. The sensitivity and reproducibility of the method was optimized through solvent and spot size studies. Calibration curves were constructed and used to evaluate the limits of detection for the method. The work shows the potential for PS-MS to be utilized as a "first line of defense" screening technique for those highly exposed to PFAS on a regular basis. It is ideal for firefighters to have routine and regular testing, and PS-MS provides a cost-effective alternative to traditional LC-MS assays. Future work will focus on improving the variability of the method by incorporating more isotopically labeled internal standards, as well as analyzing realistic samples from individuals highly exposed to PFAS through their occupation.

4.6 References

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CHAPTER 5. BLOW FLIES AS REMOTE SAMPLING DEVICES: DETECTION OF INSENSITIVE MUNITIONS IN THE ENVIRONMENT USING LC-MS

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5.1 Abstract

In this work, blow flies were investigated for their use as environmental sensors of insensitive munitions contamination in the environment. Blow flies are constantly sampling their surrounding environment in search of food and water, traveling 10s of kilometers towards a nourishment source. Therefore, they were selected for this study due to their ability to cast a wide sampling net in a potentially contaminated environment. This work focused on initial feeding experiments showing the feasibility to detect insensitive munitions (IM) in the flies after exposure to contaminated environmental sources. A liquid chromatography mass spectrometry (LC-MS) method was developed to detect IM components and their transformation products in fly matrix. Flies were exposed to the IM components, 2,4-dinitroanisole (2,4-DNAN), nitroguanidine (NQ) and nitrotriazolone (NTO) in controlled feeding experiments. After exposing the flies to IMs in soil (100 µg/g (DNAN)/200 µg/g (NTO and NQ)), it was found that the components were detected in whole flies at these levels. Additionally, after exposure to the parent IM component 2,4-DNAN, the transformation product 2,4-dinitrophenol (2,4-DNP) was also detected in the fly samples. The preliminary work outlined in this chapter indicates that blow flies could be a potentially useful organism for sampling the environment for IM contamination and transformation.

5.2 Introduction

The defense industry has moved away from using shock sensitive explosives in favor of insensitive munitions (IM) which are less susceptible to unintentional detonation[1]. When using traditional explosives, a small fire, or accidental discharge can set off a chain reaction leading to a

large number of casualties and significant damage to property. For example, in 1967 there was an accidental rocket launch on the U.S.S. Forrestal supercarrier that set off a large fuel fire[2]. This accident led to the deaths of many service members following the detonation of the cargo containing traditional explosives. In response to this and other similar incidents, the U.S. military prioritized the development of new explosives that would not as easily detonate in the event of combat threats, blast threats, or fuel fires[3].

Insensitive munitions are often composed of mixtures of multiple constituents. One of the most popular IM formulations is IMX-101 which was developed by BAE Systems and the U.S. Army to replace traditional high energy explosives such as 2,4,6-trinitrotoluene (TNT) in artillery rounds[4]. IMX-101 contains 2,4-dinitroanisole (DNAN), nitrotriazolone (NTO), and nitroguanidine (NQ). Additionally, IMX-104, composed of DNAN, NTO and royal demolition explosive (RDX), is being used to replace Composition B which is a combination of TNT and RDX[5, 6].

While IM-based chemistries are safer to transport than more conventional explosives, due to incomplete detonation and high water solubility of constituents, they may pose a higher risk to the environment[6]. TNT, and its environmental impact, have been studied extensively over the last 40+ years, therefore much is known about the fate, transportation, and toxicity risk to the environment[7-12]. IM formulations were implemented fairly recently in comparison, therefore not as much is known about their potential harm post-detonation. The major constituents of insensitive munitions, NTO, NQ, DNAN and RDX, have water solubilities of 16,642[13], 5000[14], 0.216[15], and 56 mg/L[15] respectively, indicating that NTO and NQ would be highly soluble in groundwater and not adsorbed to soil upon release into the environment.

Recent works have identified biotic and abiotic transformation products for many of the major IM constituents. For example, DNAN is degraded to 2-amino-4-nitroanisole (2-ANAN) and 2,4-diaminoanisole (2,4-DAAN) under aerobic and anaerobic conditions, respectively[16]. Under photolysis, DNAN degrades to 2-hydroxy-4-nitroanisole and 2,4-dinitrophenol (2,4-DNP), which are considered eco-toxic transformation products [16, 17]. NTO degrades primarily to 3-amino-1,2,4-triazol- 5-one (ATO) and urazole (UZ)[18, 19]. Finally, nitroguanidine degrades to cyanoguanidine (CQ)[20]. Many studies focus on degradation in soil/water matrices, however, this type of sample requires sending personnel into the field to collect samples. Oftentimes these locations are remote, inaccessible, or dangerous, so remote sampling would be advantageous. This

work offers a potential solution by using insects as environmental samplers of IM contamination and transformation.

Blow flies (Diptera: Calliphoridae) are well-suited to address the issue of sampling since they are constantly sampling their surrounding environment[21]. They are found on all continents except for Antarctica, and in a variety of climates and ecosystems. Due to their scavenging nature, they are constantly in search of sustenance, and will travel kilometers to a food/water source. For example, *Phormia regina* has been recorded to travel up to 45 kilometers from a release point[22]. Previous work has shown that blow flies harbor chemical and biological information, such as DNA and fecal metabolites in their stomach contents after exposure[23, 24].

In a recent project published in 2022 in Environmental Science and Technology, blow flies were utilized as chemical sensors of chemical warfare agent (CWA) release[25]. In this work, blow flies were exposed to chemical warfare agent simulants and then subjected to a variety of temperature and humidity conditions. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was developed to detect chemical warfare agent simulants, CWA hydrolysis products, and organophosphorus pesticides. Chemical signatures were detected in the fly guts up to 14 days post-exposure indicating a long window of detectability for these chemicals. Additionally, the chemical warfare hydrolysis product, isopropyl methylphosphonic acid (IMPA), was detected in the fly gut after feeding the fly diisopropyl methylphosphonate (DIMP), indicating that not only are the flies consuming the chemicals but they are transforming them metabolically.

In this project, blow flies were shown to harbor insensitive munition chemical signatures after exposure to a contamination source. Two LC-MS methods, hydrophilic interaction chromatographic (HILIC) and reversed phase (RP), were developed to detect the IM components and allows for retrospective analysis of the fly samples for transformation products. While the previous work focused on controlled feeding experiments from a Kimwipe, this work investigated the interaction between flies and contaminated soil and water matrices. The overall goal of this project was to develop an analytical technique to detect IM and their transformation products in whole blow flies, as well as, understand the longevity of these chemicals in the flies using controlled feeding experiments and more realistic environmental samples.

5.3 Experimental Methods

5.3.1 Chemicals and Materials

High-performance liquid chromatography (HPLC) grade acetonitrile, HPLC grade methanol, HPLC grade water, and ammonium acetate were purchased form Fisher Scientific (Hampton, NH, USA). IM compounds: 2,4-dinitroanisole (2,4-DNAN) and nitrotriazolone (NTO) were acquired from BAE Systems (Kingsport, TN, USA). Cyanoguanidine (CQ), royal demolition explosive (RDX), 2,4-diaminoanisole (2,4-DAAN), 2-methoxy-5-nitrophenol (2,5-MNP), urazole (UZ), and nitroguanidine (NQ) were purchased from Sigma Aldrich (St. Louis, MO, USA). The isotopically labeled internal standard 2,4-dinitrophenol-d_3 and cyanoguanidine-^{13}C were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and the internal standard p-toluidine-d3 was purchased from C/D/N Isotopes Inc. (Pointe Claire, Quebec, CAN).

5.3.2 Calibrator and QC Preparation

Stock solutions were prepared individually by dissolving standards in methanol or water. The stock solutions were used to prepare a 50-µg/mL working solution in methanol containing all standards. The 50-µg/mL solution was used to create the calibrators. The final concentration range was 15 – 1215 ng/mL. A separate set of working solutions was prepared for the quality control (QC) sample at 135 ng/mL. An internal standard solution was prepared by diluting reference materials in methanol to 50 µg/mL. Prior to analysis, 2.5 µL of IS solution was spiked into the calibrators and QC sample (300 µL).

5.3.3 Blow Fly Extraction

Experimental flies were freeze-killed. For initial experiments, the fly digestive system was dissected out using flame-sterilized forceps. The dissection was omitted for later experiments and chemical analysis was instead performed using whole flies. Chemical components in the fly samples were extracted by sonicating the sample for 30 minutes in methanol (300 µL). A sample aliquot of 200 µL was transferred to an autosampler vial before being spiked with a 50 ppm internal standard solution (1.67 µL).

5.3.4 LC-MS Assay Development

Assay development was performed on an UltiMate 3000 HPLC system coupled to a Q-Exactive Focus mass spectrometer from Thermo Fisher Scientific (San Jose, CA, USA). Two separate columns were utilized in this study. The first was an XBridge BEH Amide Column (2.1x100 mm, 2.5 µm particle size) from Waters Corporation (Milford, MA, USA), with an Acquitiy Column In-Line Filter, BEH Amide XP VanGuard Cartridge (2.1 mm X 5 mm, 2.5 µm particle size), and a VanGuard Cartridge Holder (Waters). The mobile phase consisted on 95/3/2 acetonitrile/methanol/water with 10 mM ammonium acetate. The 10-minute method was run isocratically. The second method used a Hypersil GOLD C18 column (100 mm x 2.1 mm, 3 µm particle size) from Thermo Fisher Scientific. The mobile phase consisted of 98:2 water:methanol with 5 mM ammonium acetate (A) and 98:2 methanol/water with 5 mM ammonium acetate (B). The gradient was run at a flow rate of 0.2 mL/min as follows: 0-7 minute linear ramp from 20 to 75% B, 7-9.5 minute hold at 75 %B, then ending with a re-equilibration period of 5.5 minutes at 20% B making for a 15-minute method. For both methods, a 2 µL injection volume was used, and the column compartment and autosampler module were not temperature controlled.

Following chromatographic separation, the eluent was analyzed by the mass spectrometer, a Q-Exactive Focus from Thermo Fisher Scientific (San Jose, CA, USA). The instrument was operated with the following parameters: Full MS mode, scan range 65-700 m/z, positive (4 kV)/negative (3.5 kV) ionization polarity switching, 35,000 resolution, 12 sheath gas (arbitrary units), and s-lens set to 50 V. The mass spectrometer parameters and the component retention times are detailed in Table 5.1.

Table 5.1 Analytes included in HPLC-MS method development and assessment. The table includes the polarity, the exact mass, the retention times and the deuterated standard utilized for each analyte

Analyte	Polarity (+/-)	Exact Mass (m/z)	Retention Time HILIC (min)	Retention Time C18 (min)	Internal Standard
cyanoguanidine (CQ)	-	83.0364	3.95	1.71	CQ[^{13}C]
2,4-diaminoanisole (2,4-DAAN)	+	139.0866	1.72	2.84	Tol-d_3
2,4-dinitroanisole (2,4-DNAN)	-	183.0048	1.79	10.28	2,4-DNP-d_3
2,4-dinitrophenol (2,4-DNP)	-	183.0048	1.79	5.09	2,4-DNP-d_3
2-methoxy-5-nitrophenol (2,5-MNP)	-	168.0303	1.65	9.03	2,4-DNP-d_3
nitroguanidine (NQ)	-	103.0264	3.60	1.80	CQ[C_{13}])
nitrotriazolone (NTO)	-	129.0054	17.33	1.66	2,4-DNP-d_3
royal demolition explosive (RDX)	-	281.0487	1.52	7.30	2,4-DNP-d_3
urazole (UZ)	-	97.9994 (HILIC) 100.0151 (RP)	6.17	1.61	2,4-DNP-d_3
cyanoguanidine-^{13}C (CQ[C_{13}])	-	85.0431	3.95	1.71	
2,4-dinitrophenol-d_3 (2,4-DNP-d_3)	-	186.0236	1.79	5.09	
toluidine-d3 (Tol-d_3)	+	111.0997	1.50	8.50	

5.3.5 Analytical Performance Evaluation

Analytical performance was evaluated for both chromatographic methods to ensure method quality and reproducibility over time. Five-point calibration curves were made using serially diluted stock solutions, where the concentrations ranged from 15-1215 ng/mL in 75/25 acetonitrile/methanol (HILIC) or methanol (RP). Limits of detection (LODs) and linearity of the calibration curves was compared across the two LC column modalities. Additionally, quality control (QC) samples (135 ng/mL) were used to evaluate the inter-day variation via percent coefficient of variation (% CV) and bias associated with the method.

5.3.6 Recovery

To assess recovery, a known amount of analyte was spiked into the fly matrix before and after the extraction protocol. Percent recovery was calculated by taking the ratio of the analyte AUC divided by the internal standard of the fly samples spiked before to the fly samples spiked after extraction.

5.3.7 Detection of Transformation Products

Since the LC-MS method was untargeted, this allowed for retrospective analysis of the data. Previous work shows that flies are transforming the chemicals they are fed into new products[25]. Additionally, IMs undergo both environmental and microbial degradation in environmental matrices. Following the controlled feeding experiments, Xcalibur Qual Browser from Thermo Fisher Scientific (San Jose, CA, USA) was used to identify the presence of suspected biological and environmental transformation products.

5.3.8 Blow Fly Colony Formation

Blow flies were colonized in the “fly room” located at IUPUI. A detailed description of the conditions can be found in Chapter 2. Only *Phormia regina* were used in this study.

5.3.9 Feeding Experiments

Experiment 1: Midgut vs. Whole Fly Sample Matrix. Cohorts of N = 5 *P. regina* were exposed to water solutions of either 0.0 µg/mL (negative control) or 50 µg/mL of DNAN, NTO and NQ for four hours.

Experiment 2: Contaminated Water Exposure. Cohorts of N = 5 *P. regina* were exposed to a water solution of either 0.0 µg/mL (negative control), 50 µg/mL, or 1000 µg/mL (DNAN: 250 µg/mL) of DNAN, NTO and NQ for four hours. DNAN was at a lower concentration in the last solution because of its lower water solubility. Following exposure period, half of the flies were immediately frozen, while the remaining flies were maintained post-exposure at 25˚C and 60%RH.

Experiment 3: Contaminated Soil Exposure. Cohorts of N = 5 *P. regina* were exposed to soil/water mixtures of either 0.0 µg/g of sterilized soil (negative control), 2 µg/g, 10 µg/g, 50 µg/g, 200 µg/g (DNAN: 100 µg/g) or 1000 µg/g (DNAN: 200 µg/g) of DNAN, NTO and NQ for four hours. Following exposure period, the flies were immediately frozen. To prepare the soil, a 1-gram sample of vacuum autoclaved Richfield clay loam or sand was deposited into an individual portion cup (Table 5.2). Then, 800 µL of either water spiked with chemicals or pure water (negative control) was used to wet the soil. The soil:water slurry was allowed to equilibrate for 12 hours prior to the feeding experiment.

Experiment 4: Contaminated Soil Exposure with Adjusted Methodology. Cohorts of N = 5 *P. regina* were exposed to soil/water mixtures of either 0.0 µg/g of sterilized soil (negative control) or 200 µg/g (DNAN: 100 µg/g) of DNAN, NTO and NQ for four hours. Following the exposure, the flies were refrigerated in the soil-containing vessel to slow the flies speed. The flies were transferred to a clean Eppendorf tube and then were frozen. The frozen flies were rinsed by submersion in methanol prior to extraction and LC-MS analysis. The soil:water slurry was prepared the same way as in Experiment 3.

Table 5.2 Soil characteristics

Soil	Texture	pH	Organic Material	Sand	Silt	Clay
Richfield	Clay Loam	7.4	3.3%	30%	43%	27%
Sand	Sand	5.9	0.0%	99.2%	0.55%	0.20%

5.3.10 Data Analysis

Tracefinder v. 3.3 from Thermo Fisher Scientific (San Jose, CA, USA) was used for data analysis. Calibration curves were made by plotting the area under the curve (AUC) of the analyte chromatography peak divided by the AUC of the internal standard chromatography peak. A 1/x weighted least squares regression was used to fit the data points. Additionally, the limits of detection (LOD) were calculated by dividing the standard error of the y-intercept by the slope of the best fit line and multiplying by a factor of 3. In order for an analyte to be considered detected, the exact mass must be within 5-ppm mass error of the standard, and the retention time must be within 30 seconds of the standard.

5.4 Results and Discussion

5.4.1 Method Development

Due to the highly polar and ionic nature of the insensitive munition compounds, a HILIC column was initially selected to retain the more polar species. Ammonium acetate buffer was found to be the optimum additive to the mobile phase, compared to formic acid, because it maximized sensitivity. CQ, NQ, and 2,4-DNP increased 3-5x in intensity, while RDX and 2,5-MNP increased greater than 10-fold. An isocratic method eliminated the need for long re-equilibration times that are common of HILIC methods. The HILIC method had two limitations. 2,4-DNAN and its major transformation product, 2,4-DNP, eluted at the same retention time. Due to unavoidable demethylation of 2,4-DNAN during ionization, the DNAN also produced a peak with an exact mass of m/z 183.0048, identical to 2,4-DNP. Additionally, NTO eluted much later than the other analytes (RT ~18 minutes). We were unable to eliminate these limitations despite gradient and mobile phase optimization. A reverse phase method using a C18 column was developed to

overcome these issues. For the HILIC method, the more hydrophobic analytes, such as RDX, eluted early in the method, while the converse was true of the RP method as seen in the extracted ion chromatograms in Figure 5.1. Additionally, 2,4-DNAN and 2,4-DNP were resolved using the RP method, and the peak shape for urazole was improved as well. A limitation of the RP separation is the poor retention and chromatographic resolution for the water soluble analytes. However, the LODs for the water soluble analytes were similar between the two methods, and the unretained analytes were well-resolved in the m/z domain. The poor retention therefore did not have a deleterious impact on assay performance.

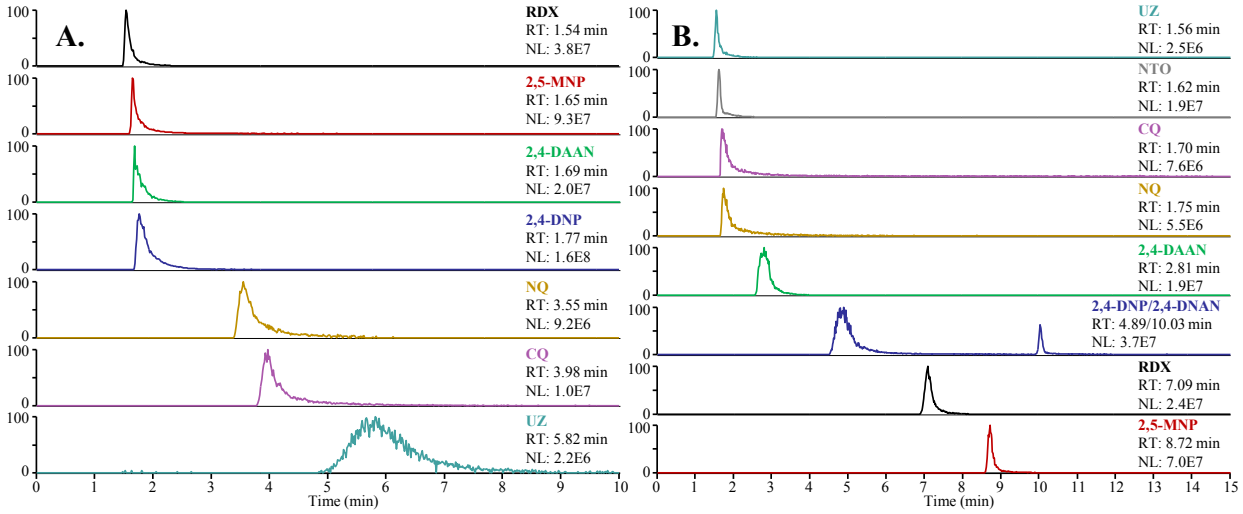

Figure 5.1 Overlayed extracted ion chromatograms of the analytes measured in the HILIC (A) and RP LC-MS (B) methods

To evaluate the analytical performance of the assays, calibration curves and QCs were analyzed across 6-days. Tables 5.3 and 5.4 outline the performance of the HILIC and RP methods, respectively. The limits of detection were comparable and were in the low ng range for most analytes. These values are the mass of analyte (ng) in 300 µL of methanol extraction solvent, and therefore represent the lowest mass of analyte detectable in the fly samples. The QC level was used to assess the %CV and bias of the methods. While not a formal validation, these values should typically be below 15%, and most of the values do fall around 15-20%. Additionally, the recoveries for the RP method ranged between 58 for 2,4-DAAN to 114% for NTO. The quantitative

performance of both methods was comparable. Due to the ability to separate 2,4-DNAN and 2,4-DNP, the RP method was selected for future experiments.

Table 5.3 The average LODs, the range of coefficient of determinations (R^2), inter-day bias and precision values obtained across 6 days for the HILIC method. %Bias= (grand mean of calculated concentration-nominal concentration/nominal concentration). %CV=standard deviation/mean

Compound	Avg. LOD (ng)	r^2 Range	QC	
			% CV	Bias
CQ	3.6	0.9878-0.9985	7.7	10.9
2,4-DAAN	22.0	0.5997-0.9852	25.9	4.1
2,4-DNP	3.1	0.9823-0.9996	7.7	13.8
2,5-MNP	4.9	0.9816-0.9979	3.4	8.7
NQ	5.4	0.9835-0.9983	10.0	21.8
RDX	4.8	0.9896-0.9973	16.9	14.0
UZ	17.7	0.9731-0.9888	7.3	22.6

Table 5.4 The average LODs, the range of coefficient of determinations (R^2), inter-day bias and precision values obtained across 6 days for the RP method. %Bias= (grand mean of calculated concentration-nominal concentration/nominal concentration). %CV=standard deviation/mean

Compound	Avg. LOD (ng)	r^2 Range	% Recovery	QC	
				% CV	Bias
CQ	2.9	0.9798-0.9991	91	9.2	6.3
2,4-DAAN	4.3	0.9621-0.9966	58	15.2	11.5
2,4-DNAN	4.3	0.9670-0.9970	94	7.9	3.9
2,4-DNP	2.0	0.9915-0.9992	81	10.0	-1.1
2,5-MNP	3.1	0.9870-0.9983	88	8.3	-2.0
NQ	3.4	0.9843-0.9974	110	9.9	0.07
NTO	4.3	0.9706-0.9946	114	14.6	29.1
RDX	9.6	0.5676-0.9963	90	10.2	7.1
UZ	7.6	0.9688-0.9969	108	10.3	-2.5

5.4.2 Feeding Studies

Previous works using blow flies to collect chemical signatures focused on midgut dissection to both extract the portion of the fly most likely to contain the chemical and to identify whether the flies were consuming the chemicals. However, midgut dissection is a specialized technique that would be difficult to implement for routine analysis. We assessed the difference in analyte intensity in the whole flies versus the fly midgut. When flies were fed 50 µg/mL of DNAN, NTO and NQ, the detected concentration in the whole fly was an order of magnitude higher or more. Additionally, more flies were positive for chemicals when analyzing the whole flies than the midguts. The intensity of the total ion chromatograms (positive and negative mode) for negative control flies were approximately three times higher when analyzing the whole fly versus the fly gut, but there was no interference in the extracted ion chromatograms at the exact mass and retention time for the analytes.

To assess the how quickly IM were cleared by the blow flies, the flies were fed either a low (50 µg/mL) or high (1000 µg/mL for NTO/NQ and 250 µg/mL for DNAN) dose. Half of the flies were frozen for analysis after the 4-hour exposure period. The other half of the flies were kept

at ambient conditions for 7-days post-exposure. No IM munitions were detected 7-days post. Table 5.5 shows amount of IM detected for the 0-day flies.

Table 5.5 Results from contaminated water feeding experiment. Each trial (T) represents an individual fly

Concentrations	**50 µg/mL**					**250/1000 µg/mL**				
Fly Replicates	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
NQ	95	151.6	181	59.2	133.8	0	0	117.2	12.3	10.4
DNAN	5.8	6.1	6.0	8.0	7.3	14	20	31.2	21	39.8
NTO	0	8	0	2.1	17.8	0	0	33.4	0	0

To simulate a more realistic sample matrix, a feeding experiment was performed where blow flies were exposed to a water:soil slurry that contained the IM components DNAN, NTO and NQ. Richfield clay loam and sand were selected for the study due to their different properties (Table 5.2).

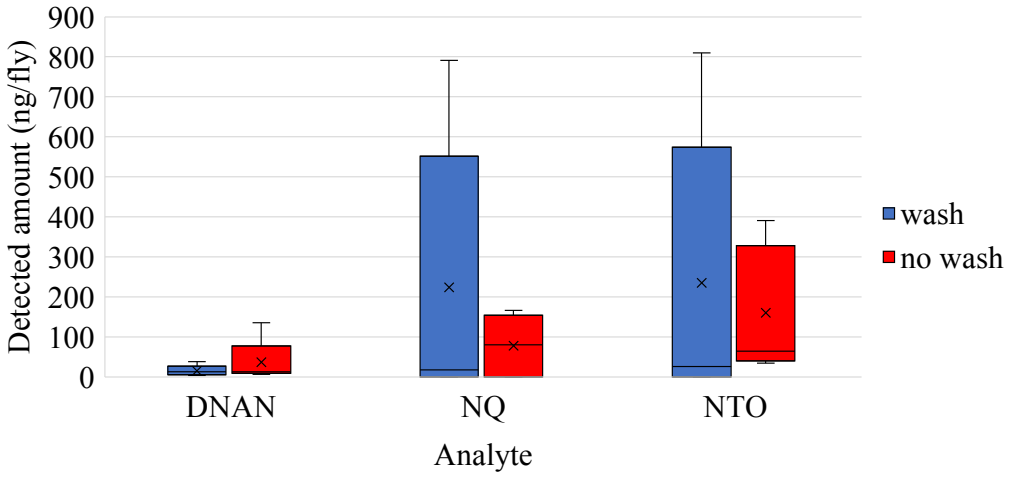

Figure 5.2 Results from the repeated preliminary soil feeding experiment where soil was spiked with varying concentrations of IM components DNAN, NQ and NTO. Flies were exposed to the contaminated soil for 4 hours before analysis. Half of the flies were rinsed with methanol prior to extraction and LC-MS analysis

In the initial soil feeding experiment, the flies were frozen in the portion cups containing the contaminated soil. Therefore, the flies were laying in the soil:water slurry when they were transferred for extraction, and high levels of chemicals were detected in the flies (thousands of nanograms per fly sample). It was hypothesized that the flies were absorbing the chemicals during the freezing process, which would not be representative of real-world conditions. Therefore, a second experiment was performed where the flies were not only transferred from their original exposure vessel before freezing, but were also washed with methanol prior to extraction. Figure 5.2 shows the results from the second soil feeding experiment. While rinsing the flies did not have a significant impact on the amounts detected in the flies, the amended sampling protocol was more realistic than the original experimental design. The US Army Regional Screening Levels (RSL) for IM components 2,4-DNAN, NTO and NQ are 20, 6300, and 2500 ppm respectively[26]. This method was able to detect IMs in the flies at 100 ppm for DNAN and 200 ppm for NTO and NQ indicating the possibility to use flies as samplers at relevant concentrations. Future work should evaluate the detectability of the chemicals following the fly exposure to different temperature and humidity conditions. However, these experiment shows the potential for IM components to be detected in the fly following environmental exposure.

5.4.3 Detection of IM Transformation Products

Insensitive munitions undergo transformation in the environment due to microbial degradation, UV exposure, and hydrolysis. Therefore, the flies that were fed DNAN, NQ, and NTO were monitored for the presence of transformation products both known and unknown. Due to previous work involving blow fly exposure to chemical signatures, it was hypothesized that the transformation products would simultaneously be detected alongside the parent compound[25]. In the top panel of Figure 5.3, a fly sample was positive for both 2,4-DNAN, which was fed to the flies via contaminated water, and 2,4-DNP which was not fed to the fly. The middle panel shows the EIC for a calibration standard containing both chemicals. The bottom panel shows the extracted ion chromatogram (EIC) for a negative control fly. The 2,4-DNAN ether bond undergoes abiotic or biological hydrolysis to form the alcohol on 2,4-DNP[27].

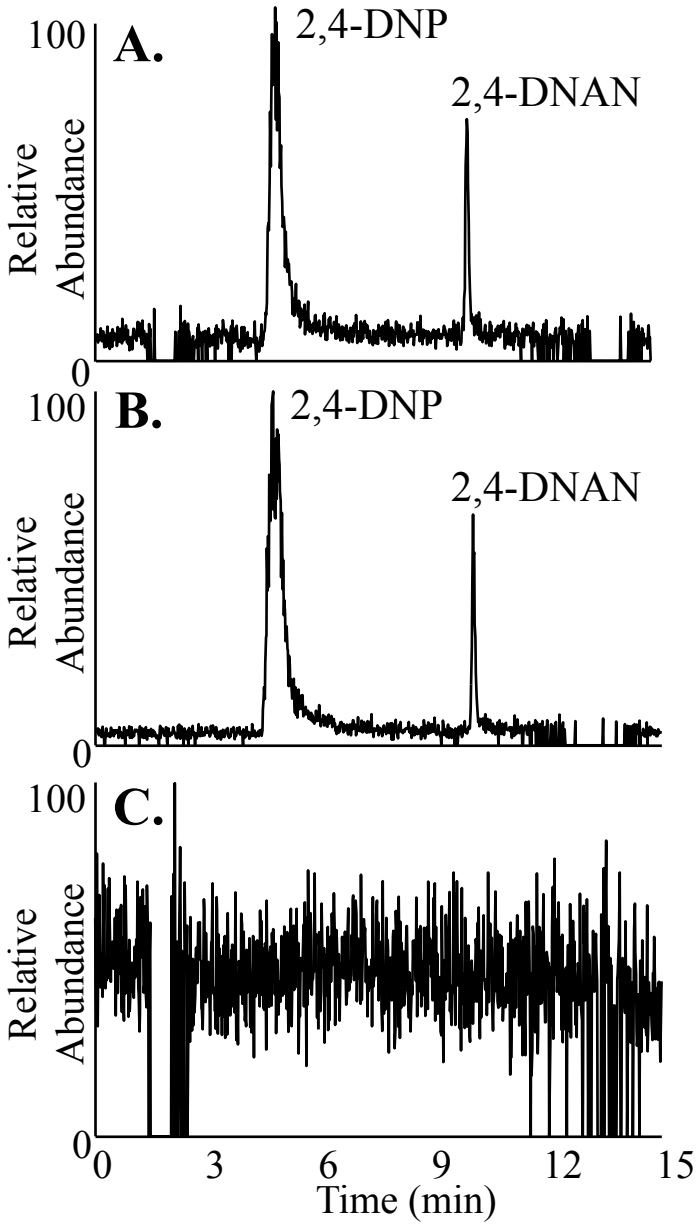

Figure 5.3 Extracted ion chromatograms (m/z 183.0048) of (A) Fly sample fed 50 µg/mL of DNAN in water, (B) 45 ng/mL calibrator, (C) Negative control fly sample

5.5 Conclusion

The data presented in this work shows the potential for blow flies to be used as environmental monitoring agents of IM contamination in the environment. Two LC-MS methods, using HILIC and RP stationary phases, were developed for the detection of insensitive munitions in the blow fly following an exposure. While both methods performed similarly, the RP was

selected because it was able to separate the insensitive munition component 2,4-DNAN and its transformation product 2,4-DNP. Three preliminary feeding experiments were performed to assess detectability in water/soil matrices, as well as compare the detectability when using the midgut or whole fly as a sample matrix. Future work should focus on the longevity or clearance of these chemicals from the flies in different temperatures and humidity conditions. This work described shows the potential for this assay to be useful to military personnel who desire a way to track IM contamination in the environment without deploying personnel for sampling.

5.6 Acknowledgements

This research was developed with funding from the Army Research Office (ARO) agreement number W911NF-22-1-0069. The views, opinions and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government.

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VITA

EDUCATION

PhD Purdue University, Indianapolis, IN August 2019 – December 2023

Focus: Analytical Chemistry

Thesis Title: Development of Mass Spectrometry-Based Analytical Assays for Environmental and Defense Applications

Advisor: Dr. Nicholas Manicke

MS Purdue University, Indianapolis, IN August 2017 – August 2019

Forensic and Investigative Sciences Program

Thesis: Detection and Quantitation of Hazardous Chemicals in Environmental Matrices using Paper Spray Mass Spectrometry

Advisor: Dr. Nicholas Manicke

BS Western Kentucky University January 2014 – May 2017

B.S. Chemistry; B.A. Criminology

Graduated Summa Cum Laude

RESEARCH EXPERIENCE

Doctoral Dissertation Work August 2019 – December 2023

Advisor: Dr. Nicholas Manicke

- Developed and validated a dual-polarity HPLC-MS/MS method to detect organophosphorus compounds in biological matrices. The project aimed to utilize blow flies as environmental sensors of chemical warfare release.
- Combined ambient ionization paper spray mass spectrometry with surface enhanced Raman spectroscopy (SERS) to detect harmful pesticides and organophosphorus nerve agents from surfaces as a combined screening and confirmatory technique.
- Developed a 1-minute assay using paper spray mass spectrometry to detect polyfluoroalkyl substances (PFAS) in whole blood without any sample preparation or clean-up. The assay can

provide a technique for screening military fire fighters who are highly exposed to PFAS through aqueous film forming foams (AFFF).

- Operated and maintained both high-resolution (orbitrap) and low-resolution (triple quadrupole) mass spectrometers using targeted and untargeted analysis.
- Developed HPLC-MS methods using hydrophilic interaction chromatography (HILIC) and reverse phase chromatographic separation.
- Mentored graduate students (4) and undergraduate students (2).

Heritage Research Group – Analytical R&D Intern. May 2023 – August 2023

Manager: Bill Gorman, Senior Analytical Research Chemist

- Assisted with summer asphalt fume testing by preparing sampling apparatuses, going to select asphalt plants, and assisting with sampling.
- Analyzed of asphalt leachate samples using untargeted liquid chromatography – evaporative light scattering – time of flight mass spectrometry.
- Performed unknown mass spectral peak identifications using Agilent Mass Hunter software.
- Updated standard operating procedures and participate in project planning meetings.
- Maintained records of experimental procedures and results.
- Presented work at analytical group meeting and final intern capstone presentation.

Heritage Research Group – Forensic R&D Intern May 2022 – August 2022

Manager: Bill Gorman, Senior Analytical Research Chemist

- Developed a normal phase, preparative chromatography method for fractionating asphalt binder to simplify a complex matrix for further analytical testing.
- Analyzed whole and fractionated asphalt samples via ATR-FTIR spectroscopy, thermogravimetric analysis, thin layer chromatography, atmospheric solids analysis probe mass spectrometry, etc.
- Kept accurate and detailed records of experimental procedures and results.
- Attended and participated in project meetings.
- Wrote SOPs and reports to be viewed and understood by fellow researchers.
- Presented findings with intern cohort and analytical team.

Master's Thesis Work August 2017 – August 2019

Advisor: Dr. Nicholas Manicke

- Developed mass spectrometry-based assays to detect drugs of abuse, chemical warfare simulants, and chemical warfare hydrolysis products in environmental matrices.
- Utilized liquid-liquid extraction, solid phase extraction, and QuEChERS.
- Maintained a TSQ Vantage and a Q-Exactive Focus mass spectrometer.

Undergraduate Research 2014 – 2017

Advisor: Dr. Rajalingam Dakshinamurthy

- Performed green synthesis of gold nanoparticles to combat multi-drug resistant bacterial infections.
- Characterized nanoparticles via UV-Vis Spectroscopy, Transmission Electron Microscopy, and Thermogravimetric Analysis.
- Evaluated antibacterial activity and mechanisms using turbidimetry assays.

TEACHING EXPERIENCE

Forensic and Investigative Sciences Teaching Assistant August 2017 - May 2018

Indiana University Purdue University Indianapolis

- Prepared and instructed senior level forensic chemistry laboratories.
- Calibrated and maintained analytical instrumentation.
- Prepared mock cases for students and assisted with mock testimony.

SKILLS

Laboratory Skills		**Computer Skills**
Mass spectrometry	Infrared Spectroscopy	Microsoft Office Products
Liquid chromatography	Instrument maintenance	Xcaliber/Tracefinder
Gas chromatography	Extraction techniques: LLE, SPE	Mass Hunter
UV-Vis spectroscopy	3D printing	mzVault
Raman spectroscopy	Microspectrophotometry	Chemstation
Electron Microscopy	Thermogravimetric analysis	Sketchup

PUBLICATIONS

Sarah N. Dowling and Nicholas E Manicke. "Dual-Technique Assay for the Analysis of Organophosphorus Compounds for Environmental and Chemical Defense Applications." *Green Analytical Chemistry*. September 2022.

Magnus Rydberg, **Sarah Dowling**, and Nicholas E Manicke. "Automated and High-Throughput Urine Drug Screening Using Paper Spray Mass Spectrometry." *Journal of Analytical Toxicology*. July 2022.

Sarah N. Dowling, Christine L. Skaggs, Charity G. Owings, Khadija Moctar, Christine J. Picard, and Nicholas E. Manicke. "Insects as Chemical Sensors: Detection of Chemical Warfare Agent Simulants and Hydrolysis Products in the Blow Fly using LC-MS/MS." *Environmental Science and Technology*. February 2022.

Christine L. Skaggs, Lindsey Kirkpatrick, Chao Nguyen, **Sarah Dowling**, Hannah Zimmerman, Greta Ren, and Nicholas E. Manicke. "Simultaneous optimization of paper spray substrates and solvents for hydrophilic and hydrophobic molecules." *International Journal of Mass Spectrometry*. 470, 116705. Special Issue: Recent Developments in Ambient Ionization. September 2021.

Sarah Dowling, Nicholas E. Manicke, Dan Carmany, Paul S. Demond, Ethan M. McBride, Phillip M. Mach, Elizabeth S. Dhummakupt and Trevor Glaros. "Screening and Quantitation of Fentanyl Analogs in Soil Using Paper Spray Mass Spectrometry." Government Technical Report: CCDC CBC-TR-1667. August 2021.

Sarah Dowling, Ethan McBride, Josiah McKenna, Trevor Glaros, and Nicholas E. Manicke. "Direct Soil Analysis by Paper Spray Mass Spectrometry: Detection of Drugs and Chemical Warfare Agent Hydrolysis Products." *Forensic Chemistry.* Volume 17, 100206. March 2020.

Ethan M. Mcbride, Phillip M. Mach, Elizabeth S. Dhummakupt, **Sarah Dowling**, Daniel O. Carmany, Paul S. Demond, Gabrielle Rizzo, Nicholas E. Manicke, and Trevor Glaros. "Paper spray ionization: Applications and perspectives." *Trends in Analytical Chemistry*. Volume 118, 722-730. September 2019.

Jason N. Payne, Vivek D. Badwaik, Hitesh K. Waghwani, Harsh V. Moolani, **Sarah Tockstein**, David H. Thompson, and Rajalingam Dakshinamurthy. "Development of dihydrochalcone-functionalized gold nanoparticles for augmented antineoplastic activity." *International Journal of Nanomedicine.* Volume 13, 1917-1926. March 2018.

Jason N. Payne, Hitesh K. Waghwani, Michael G. Connor, William Hamilton, **Sarah Tockstein**, Harsh Moolani, Fenil Chavda, Vivek Badwaik, Matthew B. Lawrenz, and Rajalingam Dakshinamurthy. "Novel Synthesis of Kanamycin Conjugated Gold Nanoparticles with Potent Antibacterial Activity." *Antimicrobials, Resistance and Chemotherapy*, 607. May 2016.

William Hamilton, Tulsi Modi, Hitesh Kumar Waghwani, **Sarah Tockstein**, Jason Payne, Oluwadamilola Filani, Vivek Badwaik, Monic Shah, Cathleen Webb, Matthew B. Lawrenz and Rajalingam Dakshinamurthy. "Nanotechnology's Impact on Medicinal Chemistry." *Mini-Reviews in Medicinal Chemistry*, 15, 1. May 2015.

RESEARCH PRESENTATIONS

Heritage Research Group Internship Capstone Presentation, August 2023.

Topic: "Analytical Chemistry and the Asphalt Industry" by **Sarah Dowling**, Marie Meyer, Andrea Moberly, Alexis Crawford, and Bill Gorman **(Oral)**

American Society for Mass Spectrometry Annual Meeting, June 2023.

Topic: "Blow flies as remote sampling devices: Detection of insensitive munitions and their degradation products in the environment using LC-MS" by **Sarah Dowling**, Sarah Prunty, Katie Jensen, Christine Picard, and Nicholas Manicke **(Poster & Invited Oral at Ambient Ionization Workshop)**

WKU Departmental Seminar, April 2023.

Topic: "Development of Innovative Mass Spectrometry-Based Analytical Assays to Protect the Warfighter" by Sarah Dowling **(Oral) *Invited seminar**

Heritage Research Group Internship Capstone Presentation, August 2022.

Topic: "Forensic Investigation of Asphalt Fractions" by **Sarah Dowling**, Ana Petro, Bill Gorman **(Oral)**

American Society for Mass Spectrometry Annual Meeting, June 2022.

Topic: "Detection and Quantitation of Per- and Polyfluoroalkyl Substances (PFAS) using Paper Spray Mass Spectrometry (PS-MS)" by **Sarah Dowling**, Alexis Zieminski*, Patrick Fedick and Nicholas Manicke **(Oral)**

*Undergraduate mentee

Annual IUPUI Chemistry Department Research Day, May 2022.

Topic: "Development of Mass SpectrometryBased Assays for Clinical, Forensic, and Environmental Applications" by **Sarah Dowling**, Hannah Zimmerman-Federle, Magnus Rydberg, Sarah Prunty, Alexis Zieminski, Lindsey Kirkpatrick, Nicholas Manicke **(Oral)**

Pittcon Annual Conference, March 2022.

Topic: "Paper Spray Mass Spectrometry using 3D Printed Devices for Forensic, Clinical, and Environmental Applications" by **Sarah Dowling**, Chengsen Zhang, Hannah Zimmerman, Greta Ren, Magnus Rydberg, and Nicholas Manicke **(Oral) *Invited symposium**

American Society for Mass Spectrometry Annual Meeting, November 2021.

Topic: "Fieldable, Dual-Technique Assay for the Analysis of Organophosphorus Compounds for Environmental and Chemical Defense Applications" by **Sarah Dowling** and Nicholas Manicke **(Oral)**

Annual IUPUI Chemistry Department Research Day, May 2021.
Topic: "Assessment of Blow Flies as Chemical Threat Agent Sensors using LC-MS/MS and Paper Spray MS" by **Sarah Dowling**, Christine Skaggs, Charity Owings, Khadija Moctar, Christine Picard, Nicholas Manicke **(Poster)**

American Society for Mass Spectrometry Annual Meeting, June 2020.
Topic: "Fieldable Assay for the Analysis of Organophosphorus Compounds in Flies for Chemical Defense Applications" by **Sarah Dowling**, Christine Skaggs, Charity Owings, Charles Sexton, Christine Picard, Nicholas Manicke **(Oral)**

Midwestern Universities Analytical Chemistry Conference, November 2019.
Topic: "LC-MS/MS Assay for the Analysis of Organophosphorus Compounds in Flies" by **Sarah Dowling**, Christine Skaggs, Charity Owings, Charles Sexton, Christine Picard, and Nicholas Manicke **(Poster)**

American Society for Mass Spectrometry Annual Meeting, June 2019.
Topic: "Screening of Chemical Warfare Agent Simulants and Hydrolysis Products in Soil Using Paper Spray Mass Spectrometry" by **Sarah Dowling**, Trevor Glaros, and Nicholas Manicke **(Oral)**

American Academy of Forensic Science Annual Meeting, February 2019.
Topic: "Detection of Fentanyl Analogs in Soil Via Paper Spray-Mass Spectrometry" by **Sarah Dowling**, Trevor Glaros, and Nicholas Manicke **(Oral)**

Annual IUPUI Chemistry Department Research Day, May 2018.
Topic: "Detection of fentanyl and its analogs in soil via paper spray mass spectrometry" by **Sarah Tockstein** and Dr. Nicholas Manicke **(Poster)**

WKU Student Research Conference, March 2016.
Topic: "Designing A Unique Therapeutic Agent Involving Gold Nanoparticles Capped with Cephalosporins For Potent Antibacterial Applications" by **Sarah Tockstein**, Jason Payne and Stuart Burris **(Oral)**

Southeastern Regional Meeting of the American Chemical Society, November 2015.
Topic: "Direct Aminoglycoside Coated Gold Nanoparticles Synthesis, Characterization, and Antibacterial Susceptibility Testing" by **Sarah Tockstein**, Jason Payne and Rajalingam Dakshinamurthy **(Oral)**

American Chemical Society National Meeting and Exposition, August 2015.
Topic: "Direct Aminoglycoside Coated Gold Nanoparticles Synthesis, Characterization, and Antibacterial Susceptibility Testing" by **Sarah Tockstein**, Tulsi Modi and Rajalingam Dakshinamurthy **(Oral)**

WKU Student Research Conference, March 2015. (Won Undergraduate Session)
Topic: "Direct Aminoglycoside Coated Gold Nanoparticles Synthesis, Characterization, and Antibacterial Susceptibility Testing" by **Sarah Tockstein**, Tulsi Modi and Rajalingam Dakshinamurthy **(Oral)**

Kentucky Academy of Sciences Annual Meeting, November 2014.
Topic: "Aminoglycoside Assisted Synthesis of Gold Nanoparticles and Their Antibacterial Activity" by **Sarah Tockstein**, Tulsi Modi and Rajalingam Dakshinamurthy **(Poster)**

Southeastern Regional Meeting of the American Chemical Society, October 2014.
Topic: "Single Step, Aminoglycoside Mediated Synthesis of Gold Nanoparticles with Potent Antimicrobial Activity" by **Sarah Tockstein**, Tulsi Modi and Rajalingam Dakshinamurthy **(Poster)**

AWARDS

- ASMS Graduate Student Travel Award, **2023**
- Dr. James H. Stuteville Scholarship, **2015**
- Fuse Grant Recipient, **2015**
- WKU Research Conference Winner, **2015**
- Ward C. Sumpter Scholarship Award - Western Kentucky University Departmental Awards, **2014**
- Owen W. Maloney Scholarship Award - University of Kansas Departmental Awards, **2013**

VOLUNTEER WORK

ACS Environmental Health and Sustainability Event – May 2022

Co-organized event

Indianapolis White River Clean-Up

Collaboration with Friends of the White River non-profit

30 total participants

ACS Unites for World Cancer Day – February 2022

Participated by making tie blankets

Provided blankets to the Little Red Door Cancer Agency

Science Night at Local Indiana School – February 6, 2019

Introduced grade school students to chemistry concepts

IUPUI Chemistry graduate student booth

Cabbage pH indicator and vinegar/baking soda volcano demonstrations

Approximately 250 participants

PROFESSIONAL AFFILIATIONS

- Environmental Health and Safety Committee Chair 2022 – 2023
  Indiana Local ACS Section
- American Society for Mass Spectrometry 2018 - Present
- American Chemical Society 2014 - Present