Method Development and Validation for the Rapid Detection of Organophosphates in Blood Plasma from Obese, Obese-Diabetic, and Lean Patients from Eastern North Carolina

by

Cheyenne Bowman

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Director of Thesis: Anne M. Spuches

Major Department: Chemistry

Abstract

The incidence of diabetes and rate of obesity is on the rise, along with the use of organophosphorus insecticides in the United States. Organophosphates (OP), a specific class of pesticide that is biodegradable and readily available for purchase, represent 50% of all insecticides used worldwide.[1] OPs are toxic and can cause numerous acute effects, but the health effects from low dose chronic exposure have not been thoroughly investigated. Interestingly, there have been few studies showing a correlation between the rise in organophosphate pesticide use and the elevated rates of diabetes and obesity.[2] These correlations should be more thoroughly investigated however the current methods of detection for OPs in human plasma use a time- and cost-consuming sample preparation method, which do not always yield accurate results. Thus, the need to develop and validate a sensitive, selective, and high-throughput analytical method for the accurate and precise determination of organophosphate levels in human plasma.

A simple "dilute and shoot" sample preparation has been developed along with an ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) method for

detection and quantification of OPs in blood plasma. The method was validated and standard curves have been generated revealing the limits of detection and quantification to range from 0.0660 ng/mL to 19.1 ng/mL and 0.200 ng/mL to 58.0 ng/mL respectively. The percent accuracies ranged from 0 to 262% for all organophosphates. Several patients showed detectable levels of diazinon, malathion, and terbufos. Interestingly, these patients were either obese or obese/diabetic. One obese and diabetic patient displayed both a detectable and quantifiable levels of diazinon (0.237 ng/mL), which had a limit of detection and quantification of 0.0660 and 0.200 ng/mL respectively. These results suggest that more intensive studies should be conducted on larger population of patients.

Method Development and Validation for the Rapid Detection of Organophosphates in Blood Plasma from Obese, Obese-Diabetic, and Lean Patients from Eastern North Carolina

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In Partial Fulfillment of the Requirements for the Degree

Masters of Science in Chemistry

by

Cheyenne Bowman

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Cheyenne Bowman

APPROVED BY:

DIRECTOR OF
THESIS: _____
Anne M. Spuches, PhD

COMMITTEE MEMBER: _____
Andrew Sargent, PhD

COMMITTEE MEMBER: _____
Yu Yang, PhD

COMMITTEE MEMBER: _____
Lisa Domico, PhD

COMMITTEE MEMBER: _____
James Harrington, PhD

CHAIR OF THE DEPARTMENT
OF CHEMISTRY: _____
Andrew Morehead, PhD

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

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Chapter 1: Introduction

1.1 Pesticides

Pesticides are any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest.[3] There are many different classes of pesticides, including herbicides, algaecides, fungicides, insecticides, etc. Each class of pesticide is used to target a specific pest group; for example insecticides are used to target insects. Approximately 1.1 billion pounds of pesticides are used in the U.S. annually and roughly 90% of these are used for agricultural purposes.[4]

1.2 Organophosphates

Organophosphorus insecticides or organophosphates (OPs) comprise a specific class of pesticide that is biodegradable and readily available for purchase. Insecticides represent 18% of all pesticide use worldwide and are responsible for 5% of pesticide use in the United States.[4] The Environmental Protection Agency reported in 2012 that about 20 million pounds of organophosphorus insecticides were used in the United States.[4] In addition to the widespread use in the agricultural industry, organophosphates are commonly used every day to eradicate household pests such as insects, rats, mice, and weeds. Some of the most commonly used OPs are chlorpyrifos and diazinon (Figure 1).

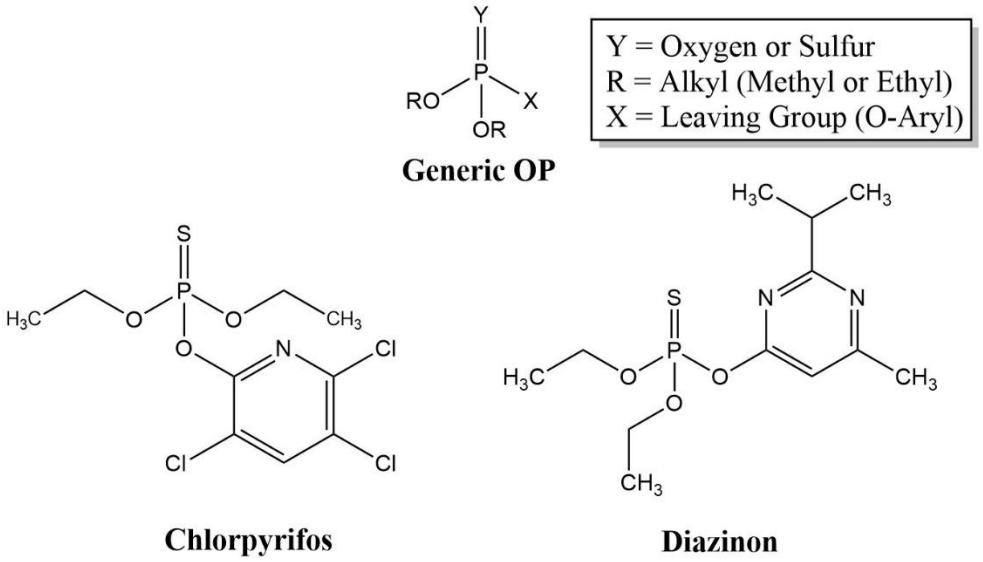

Figure 1: Structure for chlorpyrifos and diazinon

Chlorpyrifos is toxic to humans and exposure has been linked to various neurological issues and autoimmune disorders.[5] Due to these exposure effects, chlorpyrifos has almost been removed completely from commercial household products and is solely used in the agricultural industry. A map depicting the estimated use of chlorpyrifos in 2013 is shown in Figure 2.[6] Areas of heavy usage include the Midwest and South Eastern United States where farming is prevalent. Diazinon, an insecticide used in both indoor and outdoor commercial pest control, was used in the United States until was also outlawed in commercial household products in 2004 due to its toxicity.[7] Diazinon is one of the few OPs that have significant lipid solubility, which allows for fat storage and therefore delayed toxicity.[8]

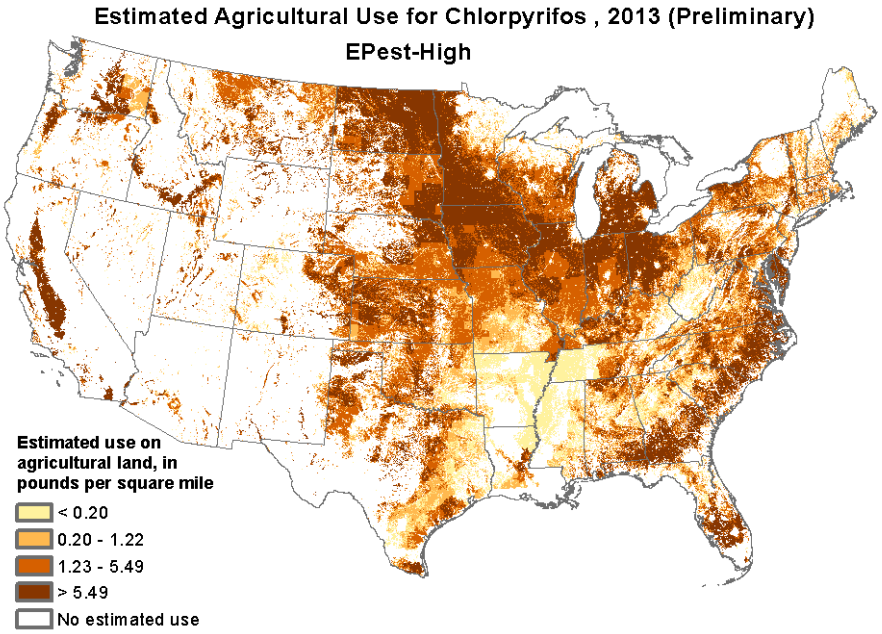

Figure 2: A map of the United States depicting estimated agricultural use of chlorpyrifos in 2013.[6]

1.3 Exposure

The main routes of exposure to OPs are oral, dermal, and/or inhalation. Human exposure can occur occupationally, from drift, and/or ingestion from contaminated food stuffs.

1.3.1 Oral

The general population is exposed to organophosphates through the ingestion of food and/or drinking water. The Environmental Protection Agency (EPA) has tolerance limits for residues of organophosphates to prevent adverse health effects. These limits vary depending on the food item. Ingestion of extensive amounts of OPs can occur deliberately or accidentally (occupational hazard).

1.3.2 Dermal

Dermal exposure occurs when handling organophosphates during either the manufacturing or application process. Dermal exposure can be varied, depending on the carrier solvent. Absorption is higher when acetone is used as a solvent compared to water. Uptake is also dependent on the hydration and temperature of the skin.[9]

1.3.3 Inhalation

Inhalation of organophosphates occurs when the pesticide is administered using sprays, mists, and powders. Pesticide applicators for example can be exposed to OPs by inhalation if not wearing proper equipment. Inhalation exposure is typically combined with exposure to the eyes and mucous membranes. Recently more than 50 farm workers were exposed to a pesticide drift in Bakersfield, California. Twelve people reported symptoms of vomiting and nausea. The active ingredient in the insecticide was chlorpyrifos, which can cause severe neurotoxic symptoms if touched, inhaled, or ingested.[10]

1.4 Toxicity

The toxicity of organophosphates is largely due to the inhibition of acetylcholinesterase by the oxon form of the OPs. Acetylcholinesterase is used to break down acetylcholine into acetate and choline. The oxon form of OPs are converted from organophosphates by cytochrome p-450. Other toxicities, however, aside from acetylcholinesterase inhibition have been studied. These include oxidative stress, delayed neurotoxicity via inhibition of NTE, mitochondrial dysfunction, and/or lipid dysfunction.

1.4.1 Cytochrome P-450

Cytochrome P-450s are a family of more than 50 enzymes that are essential for the metabolism of many toxicants and drugs. Cytochrome P-450 is predominantly expressed in the liver. Upon ingestion, organophosphates are transported to the liver through the blood stream and then metabolized in the liver by different isoforms of cytochrome P-450 and its esterase enzymes. These enzymes are known to convert OPs to their respective oxon, diethylphosphate and diethylthiophosphate metabolites (**Figure 3**).[11]

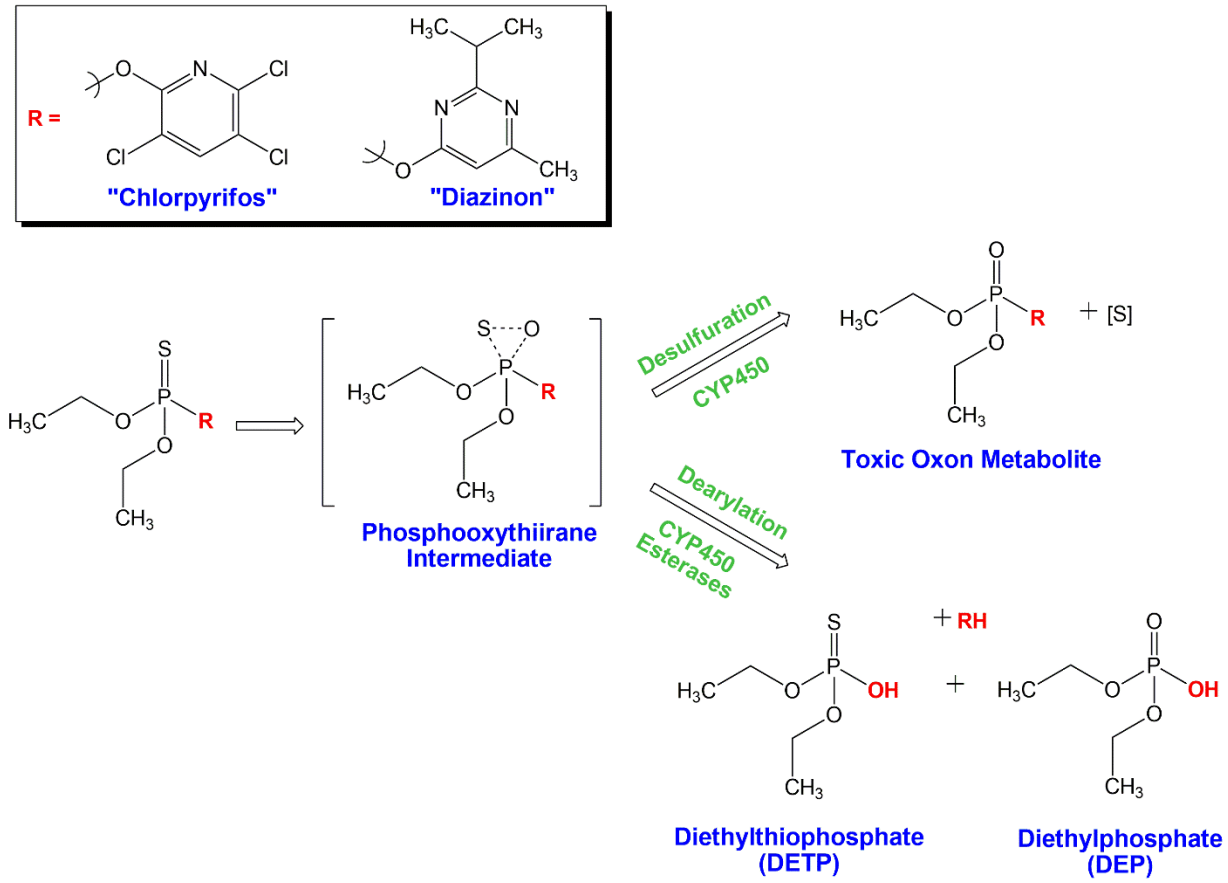

Figure 3: Metabolic breakdown of organophosphates by cytochrome P-450 and various esterases.[11]

1.4.2 Inhibition of Acetylcholinesterase

These metabolites exert their toxicity by inhibiting various enzymes in the cell. The oxon metabolite is particularly toxic because it inhibits acetylcholinesterase, an enzyme responsible for converting acetylcholine to choline and acetate (**Figure 4**).[12-13] An increase in the amount of acetylcholine in the body causes continuous stimulation of the muscles, glands, and central nervous system which can result in a variety of neurological issues and, ultimately, fatal convulsions.

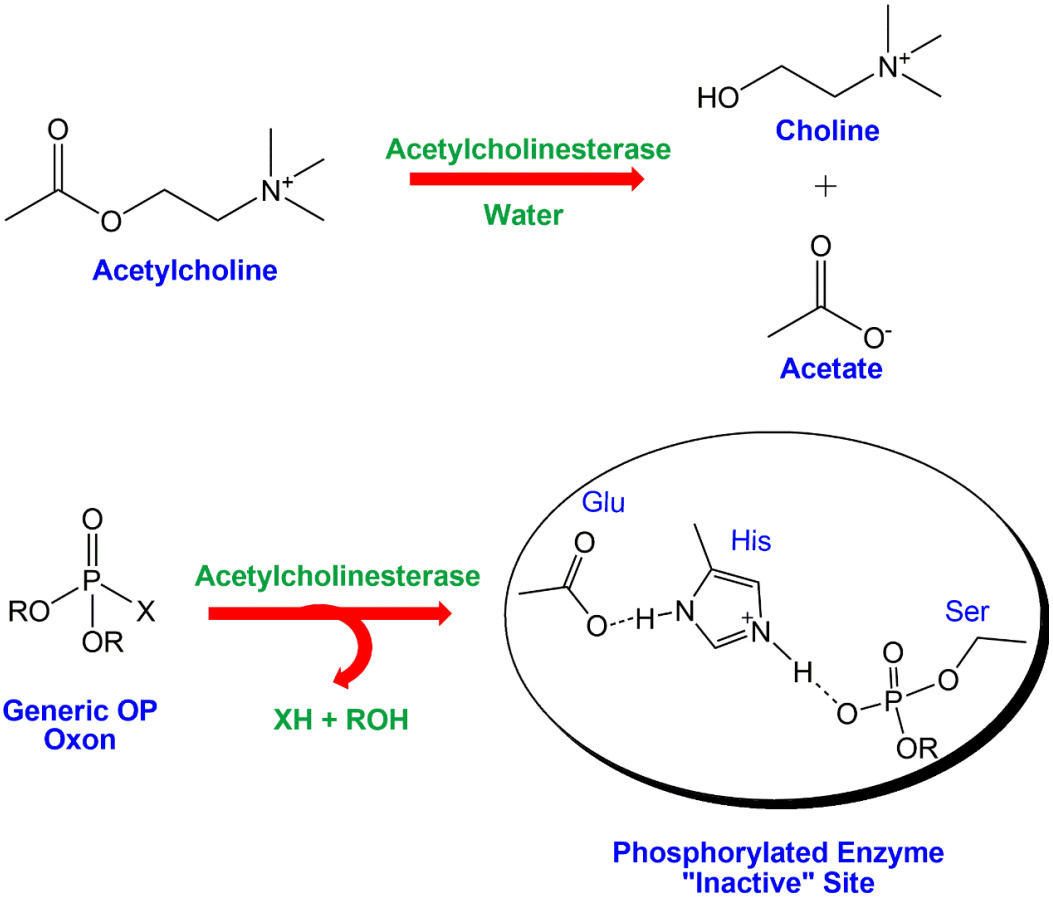

Figure 4: Acetylcholinesterase enzyme function. The top scheme represents normal acetylcholinesterase activity. The bottom scheme represents inhibition of acetylcholinesterase by a generic OP oxon metabolite.[12-13]

1.4.3 Acute and Chronic Effects

Acute OP poisoning causes a cholinergic crisis, which results in salivation, lacrimation, urination, diarrhea, gastrointestinal distress, and emesis. Symptoms can also include headaches, muscle twitching, nausea, tachycardia, and seizures. Acute effects have been heavily studied, while chronic effects have not. The long term or chronic effects from organophosphate poisoning are just recently being investigated. Neurological deficits are observed after chronic exposure to organophosphates due to the inhibition of acetylcholinesterase.[12] Chronic low dose organophosphate exposure has also been linked to the incidence of diabetes and obesity in animal and human models.[14-17]

A health study conducted from 1993 to 2003 showed licensed pesticide applicators in Iowa and North Carolina who had been exposed to insecticides had an increased risk of developing diabetes. Specifically, seven organophosphates chlorpyrifos, coumaphos, diazinon, dichlorvos, phorate, terbufos, and trichlorfon, showed increased odds of developing diabetes.[16]

Slotkin and coworkers reported in 2010 that neonatal rats who were given chlorpyrifos, diazinon, and parathion developed metabolic dysfunction which resembled prediabetes.[17] In adulthood, the rats consumed a high fat diet that led to an excessive amount of weight gain. Results of the study concluded that early-life exposure to organophosphates leads to metabolic dysfunction and a defective central nervous system that could lead to diabetes and obesity (**Figure 5**).[17] These data imply that the pathways underlying diabetes and obesity are complex and multi-factorial.

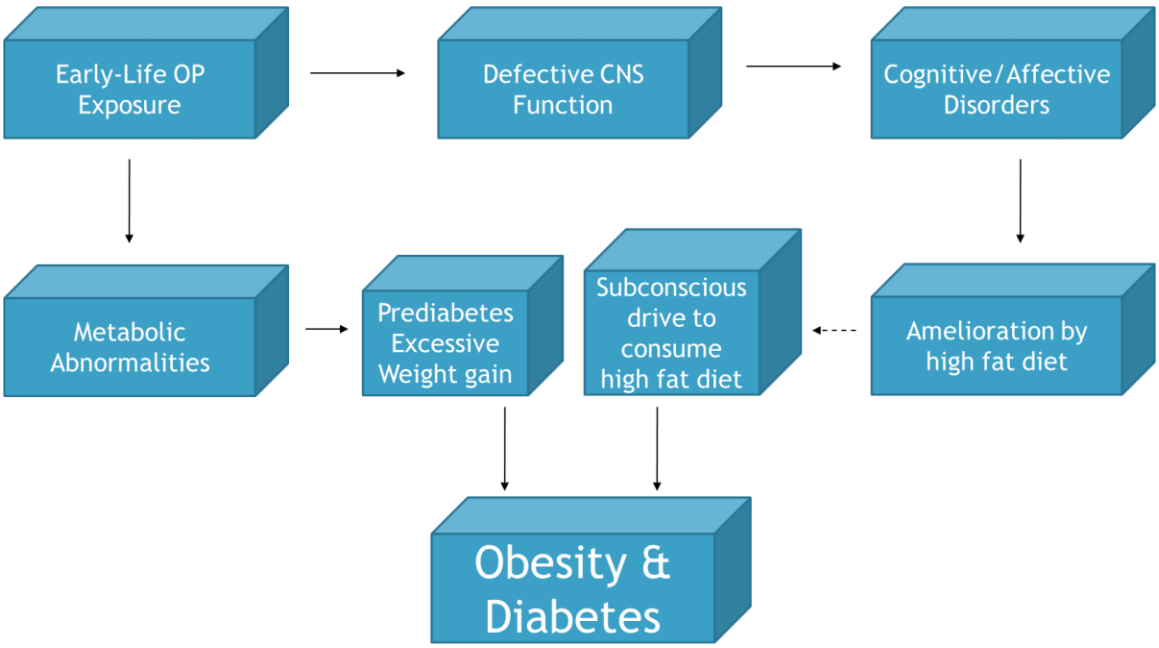

Figure 5: How early-life exposure to OPs could contribute to obesity and diabetes. Adapted from **Figure 1** in "Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity?"[17]

Currently, it is unknown how OPs contribute to the development of diabetes. Some studies, however, suggest that OPs interact with acetylcholinesterase and cause downstream toxic effects that result in a diabetic state. Type 2 diabetes is a combination of insulin resistance and β-cell dysfunction. β-cells are located in the islet, which is located in the pancreas. Pancreatic β-cells contain M_3-muscarinic receptors (acetylcholine receptors) that are responsible for regulating insulin homeostasis (**Figure 6**). One hypothesis is when organophosphates inhibit acetylcholinesterase, causing a buildup of acetylcholine in the body. The abundance of acetylcholine floods the M_3-muscarinic receptor causing an increase of insulin to be released. This causes the body to become accustomed to the high amount of insulin. The abundance of acetylcholine eventually causes the M_3-muscarinic receptor to shut down, therefore causing beta

cell dysfunction. The body has become used to the high amount of insulin causing insulin resistance. The beta cells are no longer able to release the amount of insulin needed and the body is used to a high amount of insulin, therefore the amount of insulin that is released from the beta cells does not affect the body.[16] The beta cell dysfunction and insulin resistance ultimately leads to diabetes.

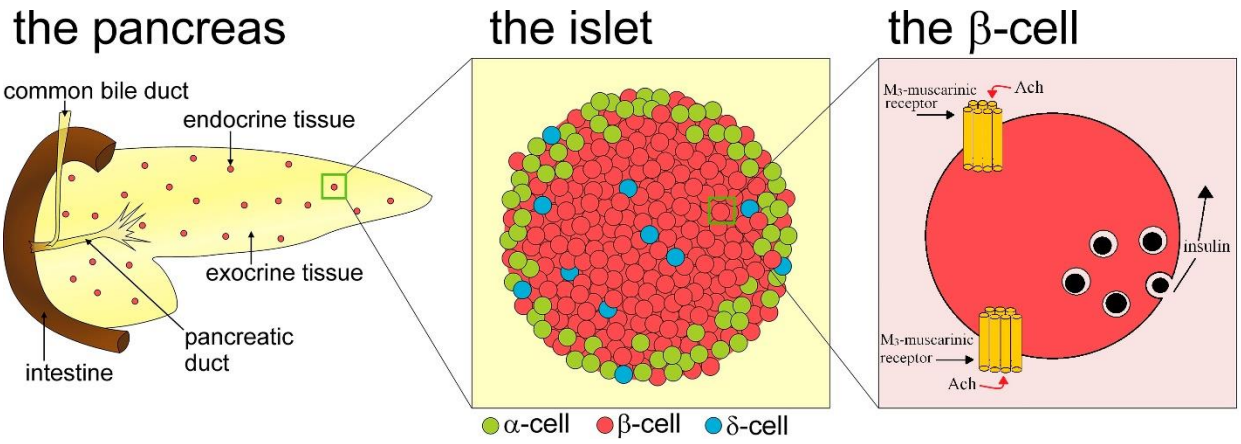

Figure 6: Cartoon depiction of the pancreas. The first panel shows the basic makeup of the pancreas. The second panel shows the makeup of an islet. The third panel shows the β-cell function in regard to producing insulin.[18]

1.5 Diabetes and Obesity

Fourteen states, including North Carolina, compose a geographic area referred to as the "diabetes belt" (**Figure 7**). [19] North Carolina has approximately 12% prevalence of diabetes, which is higher than the national average of 9.3%.[20] The diagnosis of obesity and diabetes has significantly increased from 1994 to 2013 as shown in **Figure 8A** and **8B**. When comparing **Figures 2** and **8**, there appears to be a correlation between the states with higher percentages of diagnosed diabetes and obesity and the states with heavy pesticide usage. However, **Figure 2** is

only of the pesticide of chlorpyrifos, so you must take into account the location of the pesticide use and other lifestyle factors. The correlation between diabetes, obesity, and organophosphates needs to be studied further to see if there is causal relationship and to understand the role organophosphates play in disease pathways. Due to the health effects caused by organophosphates, it is crucial to develop rapid and sensitive analytical techniques to test for OP exposure. Current analytical methods rely on liquid-liquid extractions and solid-phase extractions to isolate organophosphates.[9,22-23] These sample preparation methods take time and depend on numerous factors such as compound polarity and volatility. Therefore, a simpler and less time-consuming sample preparation method needs to be developed. It is therefore the goal of this study to develop and validate a method that meets the above criteria and to test the method on plasma samples that have been collected from actual patients. The overarching goal of the study is to assess the association between organophosphate levels in human plasma, obesity, and diabetes.

Figure 7: A map from 2013 highlighting the states that comprise the "diabetes belt". The region spans 15 states and 644 counties.[19]

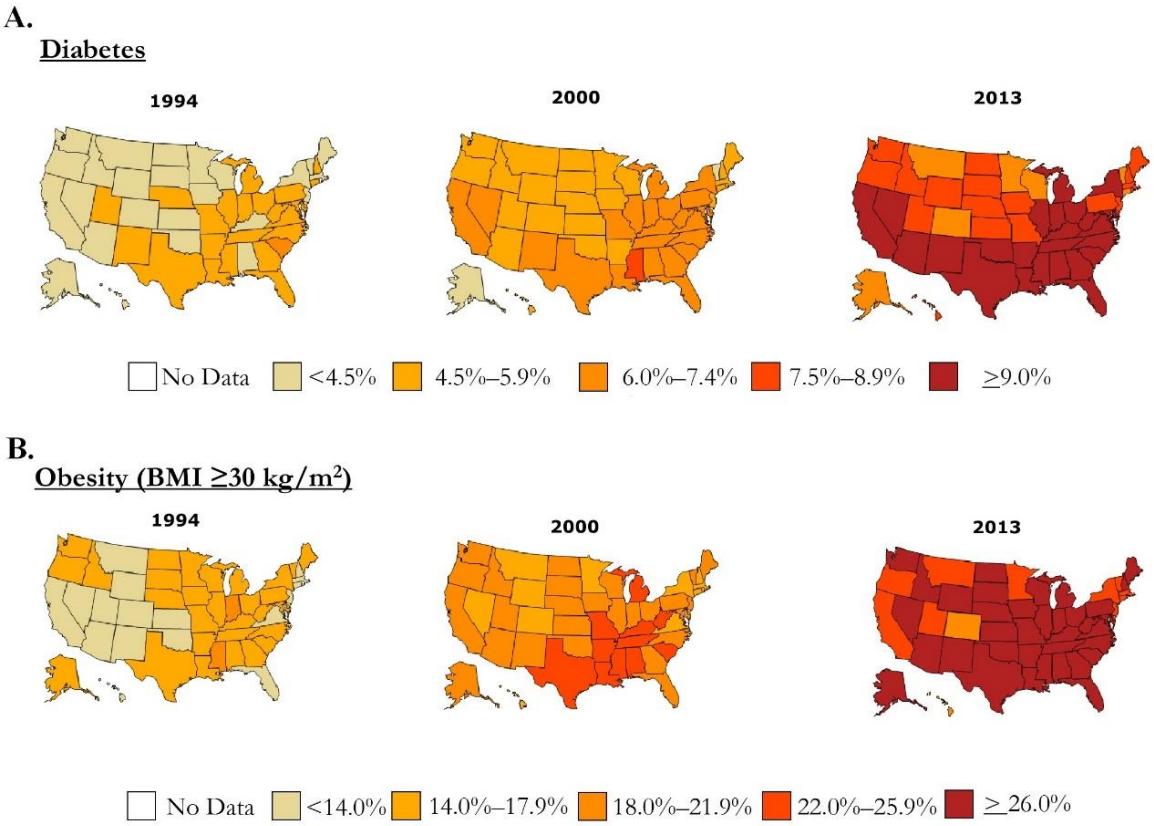

Figure 8: A) A comparison of the diagnosis of diabetes in the United States (1994, 2000, and 2013). Data reveal an increase in the incidences of diabetes from 1994 to 2013.[21] B) A comparison of the diagnosis of obesity in the United States (1994, 2000, and 2013). Data reveal an increase in the incidences of diabetes from 1994 to 2013.[21]

Chapter 2: Instrumental Methods

2.1 Previous Methods Used to Detect Organophosphates in Plasma

Organophosphate analysis is typically performed using solid phase extraction or liquid-liquid extraction followed by gas chromatography mass spectrometry or liquid chromatography mass spectrometry. Barr and coworkers analyzed 29 pesticides in human plasma of which 6 were the OPs of interest in this study. The method employed solid phase extraction followed by gas chromatography mass spectrometry. They achieved limits of detection (LOD) from 0.5 to 12 parts per trillion (ppt). Although the LODs were low, the percent recoveries for the spiked human plasma only ranged from 14 to 27%.[24] A different study conducted by Tarbah analyzed 23 OPs in human serum, 2 of which coincided with the organophosphates of interest in this study. The method employed a liquid-liquid extraction followed by gas chromatography mass spectrometry. The percent recoveries for the spiked human serum ranged from 50 to 133% of spiked human serum.[1] A study conducted by Musshoff analyzed 22 organophosphates in human whole blood, 4 of which were the OPs of interest in this study. The method employed solid phase micro extraction followed by gas chromatography mass spectrometry. The LODs ranged from 0.01 to 0.10 ppt. Although the LODs were low, the percent recoveries for the human whole blood were also low and ranged from only 0.1 to 19.6%.[25]

2.2 Dilute & Shoot

The main concept of the dilute & shoot sample preparation method is essentially diluting the sample and then injecting it into the UPLC-MS. The method is simple to employ and cost efficient. Its of the method saves sample preparation time and therefore reduces the overall

amount of time spent on the experiment. Reducing the overall time spent on the experiment also reduces the cost, compared to other sample preparation methods like solid phase and liquid-liquid extractions which require more time.

2.3 UPLC-MS

Ultra-performance liquid chromatography (UPLC) is a relatively new technique that is furthering the possibilities in liquid chromatography.[26] UPLC is similar to high-performance liquid chromatography (HPLC) in that it uses high pressure and a column to separate analytes. These analytes are then identified by either absorbance, mass to charge ratio, or counts per second depending on the detection method available. However, while HPLC columns contain particles with sizes ranging from 2 to 5 μm and maximum pressures around 6000 psi (~400 barr), UPLC systems are specially designed to use columns with particle sizes below 1.2 μm and maximum operating pressures of 15,000 psi (~1000 barr).[23] The smaller particle size and increased pressures result in better resolution, speed, and sensitivity.[27] UPLC has many advantages over traditional HPLC, such as increased sensitivity, using smaller volumes of solvents, and higher throughput due to its shorter elution time. The latter factors can result in significant cost savings for laboratories and their contract clients.

Chapter 3: Method Validation & Results

3.1 Method Overview

As previously mentioned, a simple dilute and shoot method was chosen for sample preparation along with UPLC-MS for sample analysis. Stock solutions were prepared along with spiking solutions, standards, and matrix standards. Once prepared the samples were injected into the UPLC-MS.

3.2 Validation

Validation involves the collection and evaluation of data, from beginning to the end of an experiment, that provides scientific evidence the process or method is consistently effective in yielding production quality results. Validation is necessary to prove a method is precise, accurate, and produces quality results. In this study, a mini validation occurred compared to a full-scale validation. The mini validation consisted of running a set of solvent and matrix standards and looking to see if the correlation coefficients were above 0.98.

3.3 Experimental

3.3.1 Materials

Chlorpyrifos, coumaphos, diazinon, dichlorvos, dichlorvos-d6, formic acid, malathion, terbufos, and trichlorfon were obtained from Sigma-Aldrich. The acetonitrile was received EMD Chemicals. The DI water came the onsite Hydro Picosystem. The UPLC-MS/MS was a Waters Acquity UPLC/Applied Biosystem 4000 QTrap. The column was a Waters Acquity BEH 1.7μm phenyl column.

3.3.2 Internal Standard, Mobile Phases, and Strong & Weak Wash Solution Preparation

The internal standard (IS) dichlorvos-d6 in acetonitrile (ACN), was prepared by weighing dichlorvos-d6 (0.000134g) into a 100 mL volumetric flask and diluting to volume with ACN giving a concentration of 1200 ng/mL. Mobile Phase A (MP A) 0.1% formic acid (FA) in 95/5 water/ACN was prepared by adding 1.0 mL FA, 950.0 mL DI Water, and 50.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. Mobile phase B 0.1% FA in 5/95 water/ACN was prepared by adding 1.0 mL FA, 50.0 mL DI water, and 950.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. The strong wash 0.1% FA in ACN was prepared by adding 500.0 µL FA and 500.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. The weak wash 80/20 water/ACN was prepared by adding 400.0 mL water and 100.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer.

3.3.3 Preparation of Stock Solutions

Solvent stock solutions were prepared for each organophosphate by weighing an allotted amount into either a 50-mL volumetric flask or scintillation vial (**Table 1**). The solutions were diluted to volume with acetonitrile and then mixed by inversion.

Table 1: Preparation of OP Solvent Stocks Used in this Study.

OP	Vol Flask used (mL)	Wt. (mg)	Dilute to volume	Mix by inversion	Conc. (mg/mL)
Chlorpyrifos	2.5	10.23	✓	✓	4.09
Coumaphos	50	4.92	✓	✓	0.0984
Diazinon	50	5.25	✓	✓	0.105
Dichlorvos	50	6.85	✓	✓	0.137
Malathion	50	4.89	✓	✓	0.0977
Phorate	50	5.45	✓	✓	0.109
Terbufos	50	11.15	✓	✓	0.223
Trichlorfon	2.5	9.53	✓	✓	3.81

3.3.4 Spiking Solutions Preparation

A 1000x ng/mL spiking solution stock was prepared by transferring aliquots of each organophosphate to a scintillation vial, adding acetonitrile (0.025 mL), and finally vortexing the solution briefly (**Table 2**). The spiking solutions with concentration factors ranging from 0.75x to 500x ng/mL were made through serial dilution by transferring a solution aliquot to a scintillation vial, diluting with acetonitrile, and vortexing briefly (**Table 3**).

Table 2: Spiking Solution Stock Preparation (SA) (~1000x ng/mL). A Specific Aliquot of Each OP was Pipetted into a Scintillation Vial, the Solution was then Diluted with Acetonitrile (ACN) and Vortexed.

OP	Stock Conc. (mg/mL)	Transfer aliquot (mL)	Add 0.025 mL ACN	Vortex briefly	Conc. (ng/mL)
Chlorpyrifos	4.09	0.25	✓	✓	102000
Coumaphos	0.0984	2.8			27600
Diazinon	0.105	0.075			788
Dichlorvos	0.137	2.0			27400
Malathion	0.0977	1.2			11700
Phorate	0.109	2.6			28300
Terbufos	0.223	0.8			17800
Trichlorfon	3.81	0.25			95300

Table 3: Preparation of Spiking Solutions (~0.75x to 500x ng/mL). Eight Spiking Solutions were Made by Serial Dilution Starting with the Spiking Solution Stock, then Aliquoting a Specific Amount of Solution, Adding ACN, and Vortexing.

Spiking Solution	Concentration Factor	Solution to aliquot	Transfer aliquot (mL)	Add ACN (mL)	Total Volume (mL)	Vortex briefly
SA	1000x	--	--	--	--	--
SA1	500x	1000x	0.5	0.5	1.0	✓
SA2	250x	500x	0.5	0.5	1.0	✓
SA3	100x	250x	0.4	0.6	1.0	✓
SA4	25x	100x	0.25	0.75	1.0	✓
SA5	10x	25x	0.4	0.6	1.0	✓
SA6	2.5x	10x	0.25	0.75	1.0	✓
SA7	x	2.5x	0.4	0.6	1.0	✓
SA8	0.75x	2.5x	0.3	0.7	1.0	✓

3.3.5 Preparation of Standards

The solvent standards were prepared by spiking DI water with the previously made spiking solutions and adding the internal standard and acetonitrile into a 1.5 mL centrifuge tube and briefly vortexing. The solutions were then centrifuged for 10 minutes at 14000 rpm and 9°C. The supernatants were transferred to limited volume inserts in autosampler vials, mobile phase A (MPA) was added, and the standards were vortexed briefly (**Table 4**).

Table 4: Solvent Standard Preparation.

Solvent Std.	Conc. Factor	Spiking Solution	Aliquot 100 µL DI H_2O	Added 25 µL Spiking Solution	Added 25 µL IS[a]	Added 350 µL ACN[b,c]	Transferred 150 µL supernatant	Added 150 µL MPA[b]
SStd1	500x	SA1	✓	✓	✓	✓	✓	✓
SStd2	250x	SA2	✓	✓	✓	✓	✓	✓
SStd3	100x	SA3	✓	✓	✓	✓	✓	✓
SStd4	25x	SA4	✓	✓	✓	✓	✓	✓
SStd5	10x	SA5	✓	✓	✓	✓	✓	✓
SStd6	2.5x	SA6	✓	✓	✓	✓	✓	✓
SStd7	x	SA7	✓	✓	✓	✓	✓	✓
SStd8	0.75x	SA8	✓	✓	✓	✓	✓	✓
IS[a] SBLK	0x	ACN	✓	ACN ✓	✓	✓	✓	✓
SBLK	0x	ACN	✓	ACN ✓	ACN ✓	✓	✓	✓

a. Internal Standard.

b. Solutions were vortexed after this step.

c. Solutions were centrifuged at 14,000 rpm for 10 minutes (9 °C) after this step.

3.3.6 Preparation of Matrix Standards

Matrix standards were prepared the same way as the solvent standards shown in **Table 4**. The only difference between the solvent and matrix is the matrix uses plasma instead of water. The matrix standards were made using rat plasma and then human plasma.

3.3.7 Instrument Parameters

A Waters Acquity UPLC/Applied Biosystems 4000 QTrap was used as the UPLC-MS system. A Waters Acquity UPLC BEH Phenyl column (2.1 x 100mm, 1.7 µm) was used. The mobile phase A was 0.1% formic acid in 95/5 water/ACN and mobile phase B was 0.1% formic

acid in 5/95 water/ACN. The gradient started at 90% A and 10% B for 5 minutes, switched to 0% A and 100% B for 2 minutes, and then back to 90% A and 10% B for 2 minutes for a total run time of 9 minutes. The flow rate was 0.5 mL/min. The column and autosampler temperatures were 25°C and 10°C respectively. The injection volume was 5 µL. The ionization mode was ESI and was in positive mode. The data system used was AB Sciex Analyst 1.6.2.

3.4 Results

The data from the solvent and matrix standards that were run by Jen Gilliam, an analyst art RTI International, were used to prove that the dilute & shoot method was suitable to use for the analysis of OP levels in human plasma. **Figure 9** illustrates the solvent and matrix standards for chlorpyrifos and diazinon with highlighted correlation coefficients. The percent accuracies were used to further prove the dilute & shoot method was suitable for OP level analysis (**Table 5**).

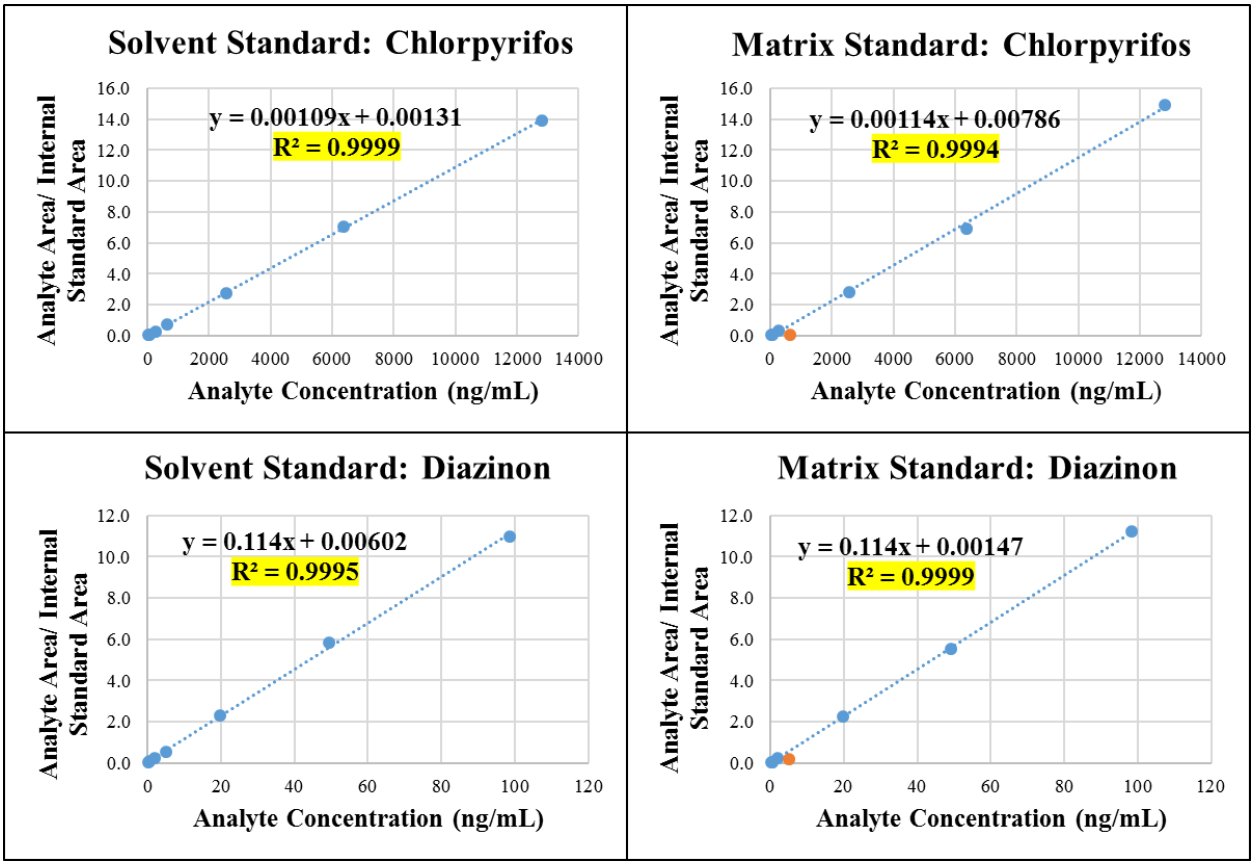

Figure 9: Solvent and matrix standards for chlorpyrifos and diazinon. Solvent standard is DI water and the matrix is human plasma.

Table 5: Solvent and Matrix Standard Percent Accuracy for Each Organophosphate

Sample Name		Analyte Peak Name	Analyte Concentration (ng/mL)		Recovered Analyte Concentration (ng/mL)		Accuracy (%)	
SA5	MA6	Chlorpyrifos	255	63.8	252	63.4	98.8	99.3
SA7	MA3	Coumaphos	6.9	690	6.87	681	99.5	98.7
SA4	MA2	Diazinon	4.93	49.3	4.85	48.7	98.3	98.7
SA4	MA1	Dichlorvos	171	3430	171	3350	100	97.7
SA4	MA2	Malathion	73.1	731	72.7	718	99.4	98.3
SA5	MA2	Phorate	70.8	1780	70.8	1750	100	98.3
SA1	MA3	Terbufos	2230	445	2220	421	99.4	94.7
SA7	MA7	Trichlorfon	23.9	23.9	23.4	23.6	98	98.5

Chapter 4: Application of the Validated Method to the Study Samples

4.1 Application of Validated Method

The validated method was applied to the study samples previously collected. The internal standards, mobile phases, stock solutions, spiking solutions, and standards were remade for the application of the validated method. The human study samples were then prepared. Once prepared, the samples were injected into the UPLC-MS.

4.2 Experimental

4.2.1 Materials

Chlorpyrifos, coumaphos, dichlorvos, dichlorvos-d6, formic acid, malathion, terbufos, and trichlorfon were obtained from Sigma-Aldrich. Diazinon was obtained from Chem-Service. The acetonitrile was received EMD Chemicals. The DI water came the onsite Hydro Picosystem. The human plasma study samples were collected from patients in Greenville, North Carolina. The UPLC-MS/MS was a Waters Acquity UPLC/Applied Biosystem 4000 QTrap. The column was a Waters Acquity BEH 1.7μm Phenyl column.

4.2.2 Human Study Samples

Volunteers for the study were recruited from East Carolina University Physicians Bariatric Clinic and Eastern Physical Medicine & Rehabilitation. Patient recruitment was done in accordance to UMCIRB 015-000984. Patients were chosen and put into the following categories: obese non-diabetic, obese diabetic, and lean. The patients were considered obese if they had a

body mass index over 30.0. A total of 45 patients were recruited, 15 for each category. Five to six mL of blood was collected into vacutainer test tubes containing K_2-EDTA. The tubes were inverted 8 times, put on ice for 15 minutes, and then centrifuged at 2500 rpm for 12 minutes. The plasma was then aliquoted into 1.5 mL microcentrifuge tubes and stored in a -80°C freezer immediately.

4.2.3 Internal Standard, Mobile Phases, and Strong & Weak Wash Solution Preparation

The internal standard (IS) dichlorvos-d6 in acetonitrile (ACN), was prepared by weighing dichlorvos-d6 (0.000134g) into a 100 mL volumetric flask and diluting to volume with ACN giving a concentration of 1200 ng/mL. Mobile Phase A (MP A) 0.1% formic acid (FA) in 95/5 water/ACN was prepared by adding 1.0 mL FA, 950.0 mL DI Water, and 50.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. Mobile phase B 0.1% FA in 5/95 water/ACN was prepared by adding 1.0 mL FA, 50.0 mL DI water, and 950.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. The strong wash 0.1% FA in ACN was prepared by adding 500.0 µL FA and 500.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer. The weak wash 80/20 water/ACN was prepared by adding 400.0 mL water and 100.0 mL ACN to a mobile phase bottle and mixed using a magnetic stirrer.

4.2.4 Preparation of Stock Solutions

Solvent stock solutions were prepared for each organophosphate by weighing an allotted amount into either a 50-mL volumetric flask or scintillation vial (**Table 6**). The solutions were diluted to volume with acetonitrile and then mixed by inversion.

Table 6: Preparation of Solvent Stocks

OP	Vol Flask used (mL)	Wt. (mg)	Dilute to volume	Mix by inversion	Conc. (mg/mL)
Chlorpyrifos	2.5	10.24	✓	✓	4.10
Coumaphos	50	4.88	✓	✓	0.0976
Diazinon	50	5.83	✓	✓	0.117
Dichlorvos	50	7.71	✓	✓	0.154
Malathion	50	5.43	✓	✓	0.109
Phorate	50	5.74	✓	✓	0.115
Terbufos	50	12.98	✓	✓	0.260
Trichlorfon	2.5	9.66	✓	✓	3.86

4.2.5 Spiking Solutions Preparation

A 1000x ng/mL spiking solution stock was prepared by transferring aliquots of each organophosphate to a scintillation vial, adding acetonitrile (0.025 mL), and vortexing the solution briefly (**Table 7**). The spiking solutions with concentration factors ranging from 0.75x to 500x ng/mL were made through a serial dilution by transferring a solution aliquot to a scintillation vial, diluting with acetonitrile, and vortexing briefly (**Table 3**).

Table 7: Spiking Solution Stock Preparation (SA) (~1000x ng/mL). A specific Aliquot of Each OP was Pipetted into a Scintillation Vial, the Solution was then Diluted with Acetonitrile (ACN) and Vortexed.

OP	Stock Conc. (mg/mL)	Transfer aliquot (mL)	Add 0.025 mL ACN	Vortex briefly	Conc. (ng/mL)
Chlorpyrifos	4.10	0.25	✓	✓	102500
Coumaphos	0.0976	2.8			27300
Diazinon	0.117	0.075			878
Dichlorvos	0.154	2.0			30800
Malathion	0.109	1.2			13100
Phorate	0.115	2.6			29900
Terbufos	0.260	0.8			20800
Trichlorfon	3.86	0.25			96500

4.2.6 Preparation of Standards

The solvent standards were prepared by spiking DI water with the previously made spiking solutions and adding the internal standard and acetonitrile to a 1.5 mL centrifuge tube followed by vortexing briefly. The solutions were then centrifuged for 10 minutes at 14000 rpm and 9°C. The supernatants were transferred to limited volume inserts in autosampler vials, mobile phase A (MPA) was added, and the standards were vortexed briefly (**Table 4**).

4.2.7 Preparation of Human Study Samples

The study samples (human plasma) were prepared the same way as the solvent standards shown in **Table 4**. The study samples were aliquoted instead of water and an additional 25 µL of ACN was used since the samples did not need to be spiked with the spiking solution.

4.2.8 Instrument Parameters

A Waters Acquity UPLC/Applied Biosystems 4000 QTrap was used as the UPLC-MS system. A Waters Acquity UPLC BEH Phenyl column (2.1 x 100mm, 1.7 μm) was used. The mobile phase A was 0.1% formic acid in 95/5 water/ACN and mobile phase B was 0.1% formic acid in 5/95 water/ACN. The gradient started at 90% A and 10% B for 5 minutes, switched to 0% A and 100% B for 2 minutes, and then back to 90% A and 10% B for 2 minutes for a total run time of 9 minutes. The flow rate was 0.5 mL/min. The column and autosampler temperatures were 25°C and 10°C respectively. The injection volume was 10 μL. The ionization mode was ESI and was in positive mode. The data system used was AB Sciex Analyst 1.6.2.

4.3 Generation of Calibration Curves and Limits of Detection & Quantification Determination

Table 8 displays the solvent standard information used to construct the calibration curves for chlorpyrifos and diazinon in **Figure 10**. The calibration curves were used to calculate the linear equations with a 1/x weighting. The linear equations were then used to determine the OP levels of the study samples.

Table 8: Solvent Standards Chlorpyrifos & Diazinon Analyte Concentrations (ng/mL) and Area Ratio.

Chlorpyrifos				
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)[a]	Area Ratio
SStd8	19.2	3.59×10^{3}	3.06×10^{5}	1.17×10^{-2}
SStd7	25.6	7.37×10^{3}	3.38×10^{5}	2.18×10^{-2}
SStd6	64.1	1.45×10^{4}	2.69×10^{5}	5.40×10^{-2}
SStd5	256	4.19×10^{4}	2.51×10^{5}	1.67×10^{-1}
SStd4	641	1.57×10^{5}	3.12×10^{5}	5.05×10^{-1}
SStd3	2560	4.14×10^{5}	2.85×10^{5}	1.45
SStd2	6410	1.01×10^{6}	3.12×10^{5}	3.25
SStd1	12800	2.07×10^{6}	3.19×10^{5}	6.48
Diazinon				
SStd8	0.165	1.52×10^{3}	3.06×10^{5}	4.95×10^{-3}
SStd7	0.22	2.23×10^{3}	3.38×10^{5}	6.60×10^{-3}
SStd6	0.549	3.66×10^{3}	2.69×10^{5}	1.36×10^{-2}
SStd5	2.2	1.08×10^{4}	2.51×10^{5}	4.30×10^{-2}
SStd4	5.49	3.49×10^{4}	3.12×10^{5}	1.12×10^{-1}
SStd3	22	7.79×10^{4}	2.85×10^{5}	2.73×10^{-1}
SStd2	54.9	2.41×10^{5}	3.12×10^{5}	7.71×10^{-1}
SStd1	110	5.72×10^{5}	3.19×10^{5}	1.79

a. Internal standard is dichlorvos d-6

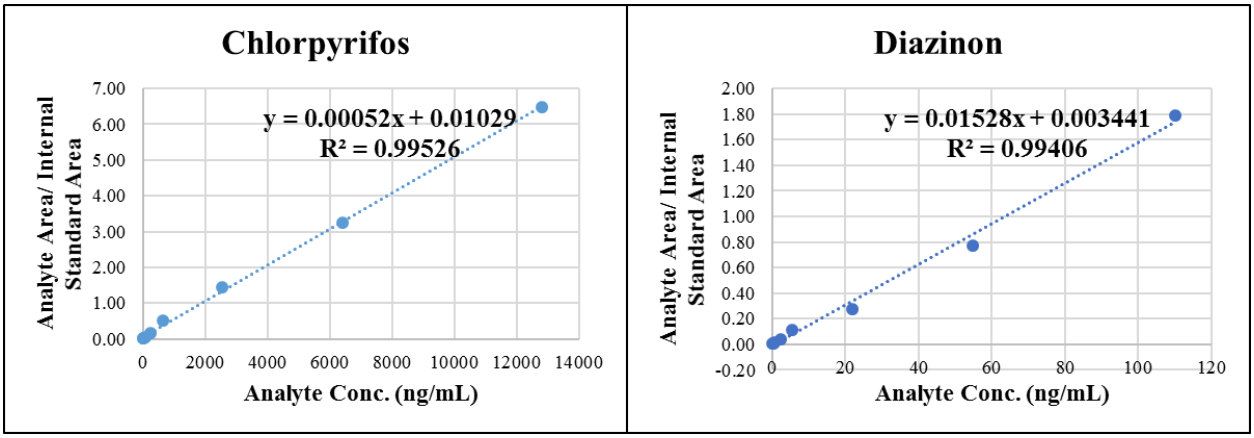

Figure 10: Solvent standards chlorpyrifos & diazinon with a 1/x weighting.

The limit of detection (LOD) is the lowest analyte concentration that can be reliably distinguished from the limit of blank (LOB). The limit of quantification (LOQ) is the lowest concentration that can reliably detected. The LOD can be calculated a few different ways such as the following:

$$LOD = LOB + 1.645(SD) \quad \text{Eq. 1}$$

$$LOD = Mean\ blank + 3.3 \times SD\ blank \quad \text{Eq. 2}$$

$$LOD = 3.3 \times \frac{\sigma}{S} \quad \text{Eq. 3}$$

SD is the standard deviation of a low concentration sample. SD blank is the standard deviation of the blank. Sigma is shown below in equation 4 and S is the slope of the linear regression.

$$\sigma = \sqrt{\frac{1}{(n-2)}\left[\sum(y-\bar{y})^2 - \frac{[\sum(x-\bar{x})(y-\bar{y})]^2}{\sum(x-\bar{x})^2}\right]} \quad \text{Eq. 4}$$

N is the sample size. X and Y are the known x and y values. X and Y bar are the averages of the known x and ys. Equation 3 will be used to determine the LOD for this study. The LOQ can also be determined a few different ways as well:

$$LOQ = Mean\ blank + 10\times(SD\ blank) \quad \text{Eq. 5}$$

$$LOQ = 10\times\frac{\sigma}{S} \quad \text{Eq. 6}$$

Equation 6 will be used to determine the LOQ for this study. The LOD and LOQ for each organophosphate is shown below in **Table 9**.

Table 9: The Limit of Detection and Quantification of Each Organophosphate. Sigma is the Standard Error of x and y. S is the Slope of the Linear Regression.

OP	*σ*	*S*	*LOD (ng/mL)*	*LOQ (ng/mL)*
Chlorpyrifos	0.00302	0.0005204	19.1	58.0
Coumaphos	0.00360	0.003543	3.36	10.2
Diazinon	0.000306	0.01528	0.0660	0.200
Dichlorvos	0.00249	0.001162	7.07	21.4
Malathion	0.00114	0.004151	0.908	2.75
Phorate	0.00150	0.0004734	10.5	31.7
Terbufos	0.000433	0.001321	1.08	3.28
Trichlorfon	0.00143	0.0004518	10.5	31.7

Chapter 5: Results & Discussion

5.1 Solvent Standard Results

The solvent standards for each organophosphate showed promising results. Each calibration curve had a high correlation coefficient, suggesting linearity. **Figure 11** illustrates the solvent standards for chlorpyrifos and diazinon with highlighted correlation coefficients. The percent accuracies for each standard and organophosphate were determined. **Table 10** gives the percent accuracies for each of the standards for chlorpyrifos and diazinon. The percent accuracy is calculated using the following equation:

$$\%\ Accuracy = \frac{Recovered\ Analyte\ Concentration\ (\frac{ng}{mL})}{Analyte\ Concentration\ (\frac{ng}{mL})} \times 100 \qquad \text{Eq. 7}$$

The correlation coefficients and percent accuracies prove that the dilute & shoot method is suitable for analysis of organophosphates in human plasma. The linear equations generated from the calibration curves were then used to determine the concentrations of the organophosphates in the study samples.

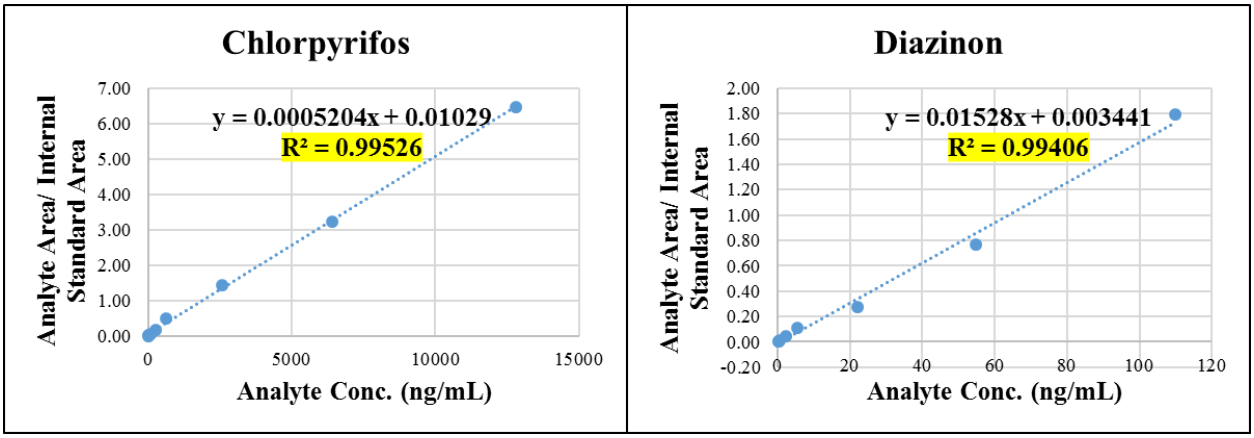

Figure 11: Solvent standards for chlorpyrifos and diazinon with a 1/x weighting.

Table 10: Percent Accuracy for Each Solvent Standard for Chlorpyrifos and Diazinon.

Sample Name	Analyte Peak Name		Analyte Concentration (ng/mL)		Recovered Analyte Concentration (ng/mL)		Accuracy (%)	
SStd8	Chlorpyrifos	Diazinon	19.2	0.165	2.78	0.0988	14.5	59.9
SStd7	Chlorpyrifos	Diazinon	25.6	0.22	22.1	0.207	86.1	93.9
SStd6	Chlorpyrifos	Diazinon	64.1	0.549	84.1	0.666	131	121
SStd5	Chlorpyrifos	Diazinon	256	2.2	301	2.59	118	118
SStd4	Chlorpyrifos	Diazinon	641	5.49	950	7.09	148	129
SStd3	Chlorpyrifos	Diazinon	2560	22	2770	17.7	108	80.3
SStd2	Chlorpyrifos	Diazinon	6410	54.9	6220	50.2	97.1	91.5
SStd1	Chlorpyrifos	Diazinon	12800	110	12400	117	97.1	106

5.2 Study Sample Results

Each of the 45 patient samples were run through the UPLC-MS and yielded interesting results. The only organophosphates detected in any of the study samples were diazinon, malathion, and terbufos. The patients that had any OP levels detected belonged to the obese or

obese-diabetic category. There were no lean patients that had any detectable OP levels. **Tables 11** through **14** give the calculated concentration organophosphate levels and the patient category.

Table 11: Patients with an Instrument Response to Diazinon and their Patient Category.

Diazinon		
Sample ID	Calculated Concentration (ng/mL)	Patient Category
OPHM 8	0.0215	Obese
OPHM 9	0.0506	Obese
OPHM 10	0.0339	Obese Diabetic
OPHM 36	0.0188	Obese
OPHM 37	0.237	Obese Diabetic
OPHM 40	0.048	Obese
OPHM 1	No Peak	-
All Others	< 0	-

Table 12: Patients with an Instrument Response to Malathion and their Patient Category.

Malathion		
Sample ID	Calculated Concentration (ng/mL)	Patient Category
OPHM 9	0.522	Obese
OPHM 37	2.41	Obese Diabetic
All Others	< 0	-

Table 13: Patients with an Instrument Response to Terbufos and their Patient Category.

Terbufos		
Sample ID	Calculated Concentration (ng/mL)	Patient Category
OPHM 8	0.0507	Obese
OPHM 9	0.0588	Obese
OPHM 37	2.49	Obese Diabetic
OPHM 38	0.141	Obese Diabetic
OPHM 40	0.628	Obese
All Others	< 0	-

Table 14: All other patients that did not Show an Instrument Response to OPs or the Levels were Below Zero

Chlorpyrifos, Coumaphos, Dichlorvos, Phorate, & Trichlorfon			
OP	Sample ID	Calculated Concentration (ng/mL)	Patient Category
Dichlorvos	OPHM 18	< 0	-
Phorate	OPHM 17	< 0	-
Chlorpyrifos, Coumaphos, Dichlorvos, Phorate, & Trichlorfon	All Others	No Peaks	-

5.3 Discussion of Results

Many the study samples did not yield any peaks for any of the organophosphates, but a few samples did such as OPHM 37. The study sample OPHM 37 had peaks for the organophosphates diazinon, malathion, and terbufos. The calculated concentration in nanograms

per milliliter were 0.237, 2.41, and 2.49 respectively. The concentration for diazinon was above the LOD (0.0660) and LOQ (0.200), while the concentrations for malathion and terbufos were above the LOD (0.908 & 1.08), but below the LOQ (2.75 & 3.28). This study sample came from a patient who is an obese-diabetic. The study samples that gave an instrument response and concentrations were all from patients who are obese or obese-diabetics, no lean patients had any detectable levels of organophosphates. This could possibly help assess the possible correlation that organophosphates could be a factor in causing obesity and diabetes. To further help this assessment it would be best to look at lowering the concentration range, looking at the metabolites of the organophosphates, and to have a larger population to sample . If the organophosphates weren't detected, that doesn't mean the patient was not exposed to OPs. These organophosphates could have been present and metabolized. Overall the results gave some detectable organophosphate levels in human plasma that could help establish the association between OP levels and obesity and diabetes.

Chapter 6: Future Goals

6.1 Lower Concentration Range

One future direction would be to lower the concentration range of the organophosphates. The calculated concentrations from the study samples were much lower than 0.75x concentration factor, which was the smallest concentration factor in range of concentration factors. Lowering the overall concentrations of organophosphates in the solvent standards would provide a more accurate calibration curve, since it would be more tailored to the OP levels that were detected in this study.

6.2 Metabolites

As mentioned in the discussion of results, just because OP levels were not detected does not mean the metabolites of these organophosphates are not in the human body. One possible experiment would to be measure the OP metabolites levels in human plasma. The experiment would essentially be the same setup as this study just using the OP metabolites instead of the organophosphates.

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Appendix A: Validation

Table A15: Solvent & Matrix Standards Concentrations by Concentration Factors (ng/mL)

OP	500x	250x	100x	25x	10x	2.5x	x	0.75x
Chlorpyrifos	12800	6380	2550	638	255	63.8	25.1	19.1
Coumaphos	3450	1730	690	173	69.0	17.3	6.90	5.18
Diazinon	98.5	49.3	19.7	4.93	1.97	0.493	0.197	0.148
Dichlorvos	3430	1710	685	171	68.5	17.1	6.85	5.15
Malathion	1460	731	293	73.1	29.3	7.31	2.93	2.20
Phorate	3550	1780	708	178	70.8	17.8	7.08	5.33
Terbufos	2230	1110	445	111	44.5	11.1	4.45	3.35
Trichlorfon	11900	5980	2390	598	239	59.8	23.9	17.9

Table A16: Solvent Standards Chlorpyrifos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Chlorpyrifos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts)[a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	19.1	1.15E+03	3.46E+04	3.31E-02	18.3	96
SA7	25.5	1.69E+03	4.27E+04	3.95E-02	24.3	95.2
SA6	63.8	3.30E+03	3.76E+04	8.78E-02	68.6	108
SA5	255	1.18E+04	4.10E+04	2.87E-01	252	98.8
SA4	638	3.32E+04	4.54E+04	7.32E-01	661	104
SA3	2550	1.04E+05	3.80E+04	2.73E+00	2500	98.1
SA2	6380	2.76E+05	3.92E+04	7.04E+00	6450	101
SA1	12800	5.58E+05	4.02E+04	1.39E+01	12800	99.6

a. Internal standard is dichlorvos d-6

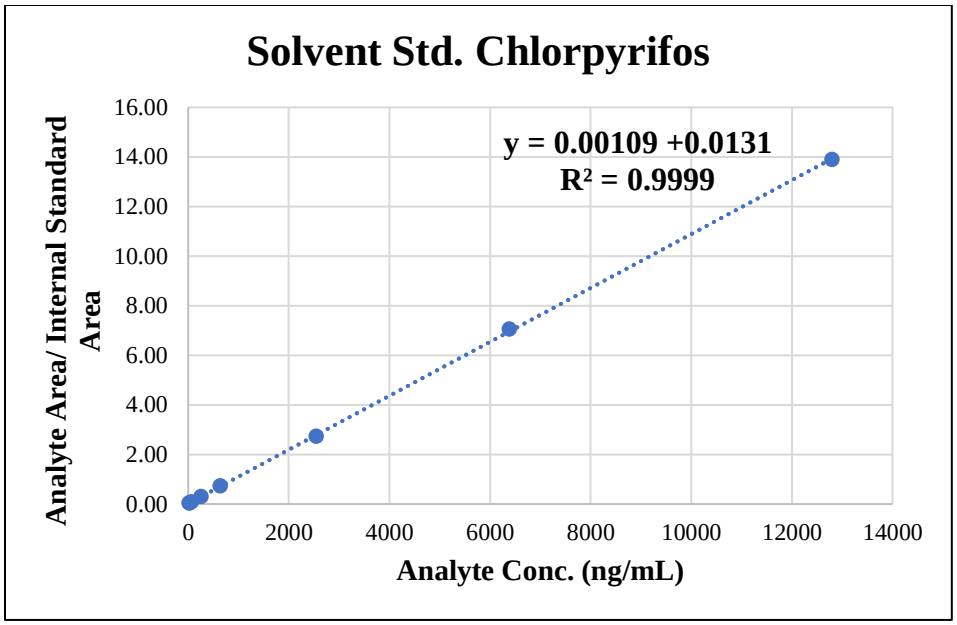

Figure A12: Solvent standards for chlorpyrifos with a 1/x weighting.

Table A17: Solvent Standards Coumaphos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Coumaphos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts)[a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.18	1.09E+03	3.46E+04	3.16E-02	5.37	104
SA7	6.9	1.56E+03	4.27E+04	3.65E-02	6.87	99.5
SA6	17.3	2.33E+03	3.76E+04	6.20E-02	14.7	84.9
SA5	69	1.05E+04	4.10E+04	2.57E-01	74.3	108
SA4	173	2.69E+04	4.54E+04	5.93E-01	177	102
SA3	690	8.49E+04	3.80E+04	2.23E+00	679	98.4
SA2	1730	2.37E+05	3.92E+04	6.04E+00	1850	107
SA1	3450	4.39E+05	4.02E+04	1.09E+01	3340	96.8

a. Internal standard is dichlorvos d-6

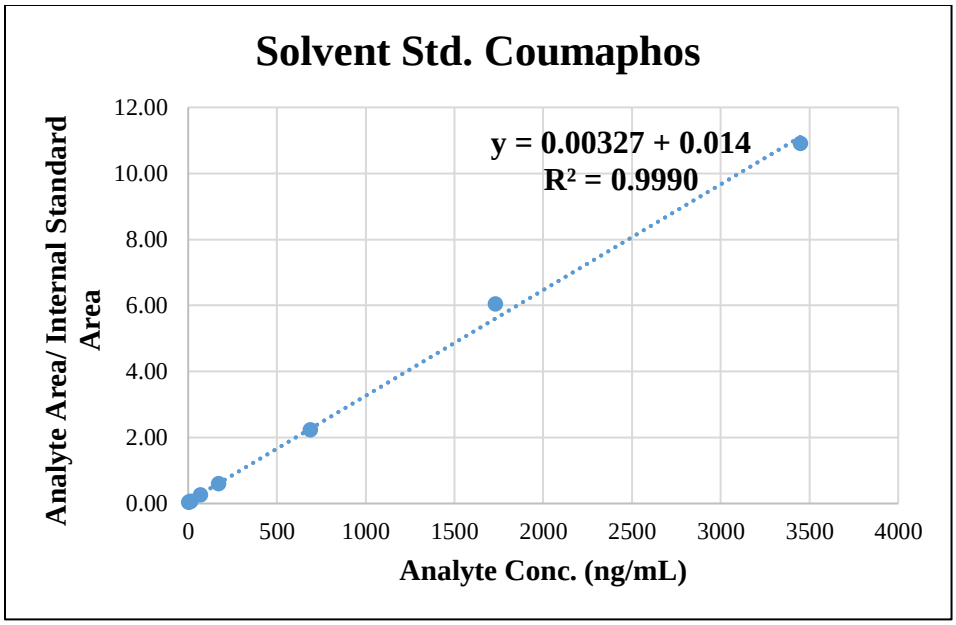

Figure A13: Solvent standards for coumaphos with a 1/x weighting.

Table A18: Solvent Standards Diazinon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Diazinon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	0.148	7.49E+02	3.46E+04	2.16E-02	0.137	92.3
SA7	0.197	1.17E+03	4.27E+04	2.74E-02	0.187	94.9
SA6	0.493	2.49E+03	3.76E+04	6.61E-02	0.525	107
SA5	1.97	9.81E+03	4.10E+04	2.39E-01	2.04	103
SA4	4.93	2.54E+04	4.54E+04	5.60E-01	4.85	98.3
SA3	19.7	8.87E+04	3.80E+04	2.33E+00	20.4	103
SA2	49.3	2.30E+05	3.92E+04	5.85E+00	51.1	104
SA1	98.5	4.41E+05	4.02E+04	1.10E+01	96	97.5

a. Internal standard is dichlorvos d-6

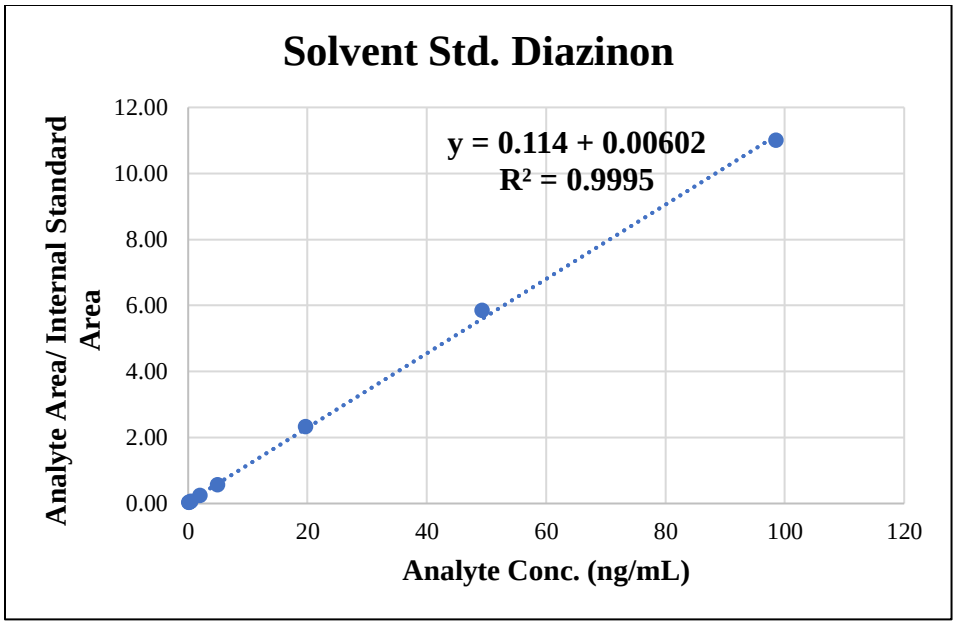

Figure A14: Solvent standards for diazinon with a 1/x weighting.

Table A19: Solvent Standards Dichlorvos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Dichlorvos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.15	7.75E+02	3.46E+04	2.24E-02	2.76	53.6
SA7	6.85	1.47E+03	4.27E+04	3.43E-02	6.54	95.5
SA6	17.1	2.56E+03	3.76E+04	6.80E-02	17.2	101
SA5	68.5	9.71E+03	4.10E+04	2.36E-01	70.5	103
SA4	171	2.52E+04	4.54E+04	5.55E-01	171	100
SA3	685	8.18E+04	3.80E+04	2.15E+00	677	98.8
SA2	1710	2.20E+05	3.92E+04	5.62E+00	1770	104
SA1	3430	4.29E+05	4.02E+04	1.07E+01	3370	98.3

a. Internal standard is dichlorvos d-6

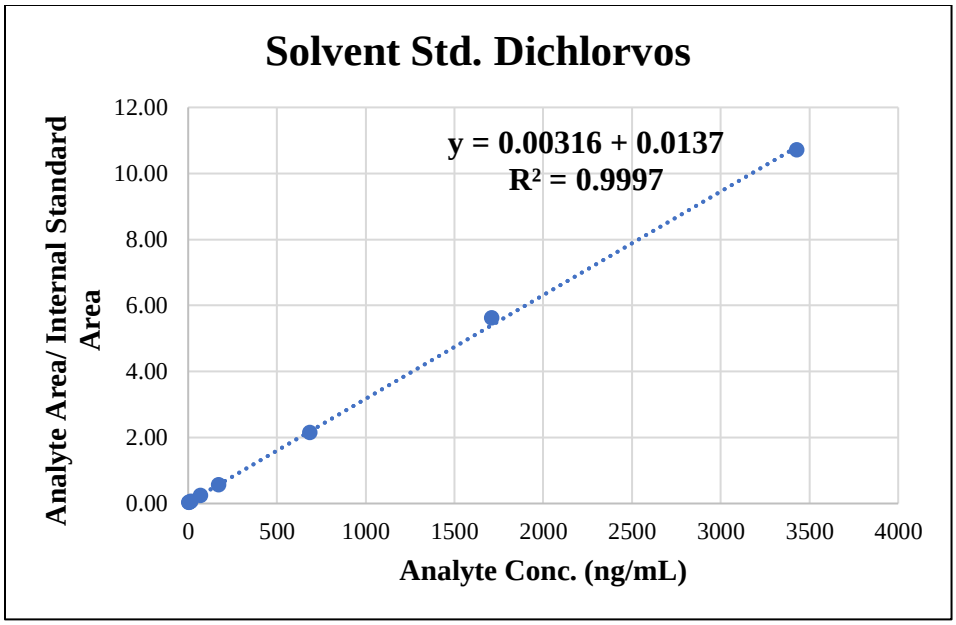

Figure A15: Solvent standards for dichlorvos with a 1/x weighting.

Table A20: Solvent Standards Malathion Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Malathion						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	2.2	9.18E+02	3.46E+04	2.65E-02	2.24	102
SA7	2.93	1.36E+03	4.27E+04	3.17E-02	2.87	98.1
SA6	7.31	2.39E+03	3.76E+04	6.36E-02	6.72	92
SA5	29.3	1.07E+04	4.10E+04	2.61E-01	30.6	104
SA4	73.1	2.76E+04	4.54E+04	6.09E-01	72.7	99.4
SA3	293	9.51E+04	3.80E+04	2.50E+00	302	103
SA2	731	2.46E+05	3.92E+04	6.28E+00	759	104
SA1	1460	4.73E+05	4.02E+04	1.18E+01	1420	97.5

a. Internal standard is dichlorvos d-6

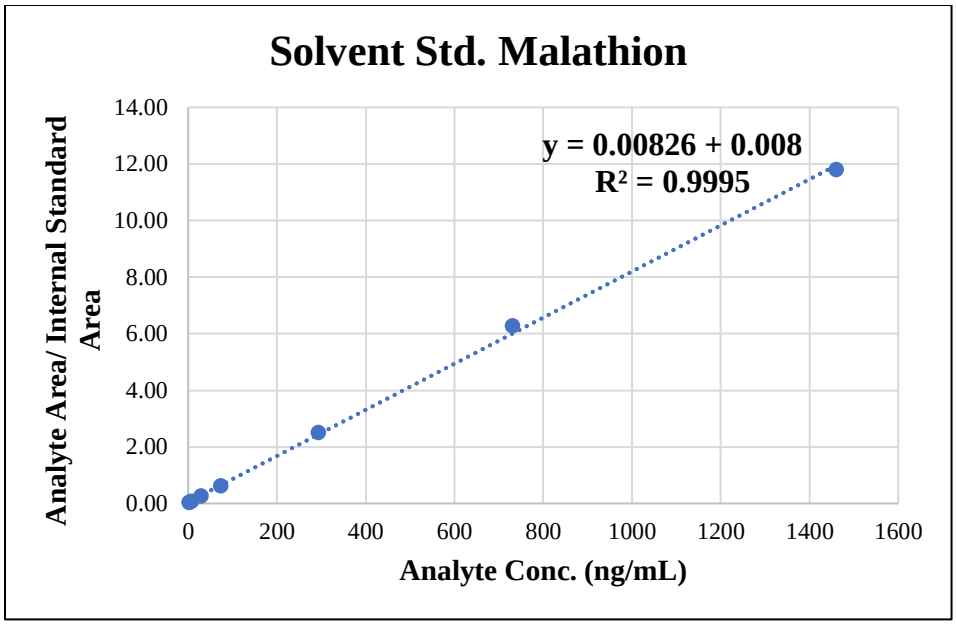

Figure A16: Solvent standards for malathion with a 1/x weighting

Table A21: Solvent Standards Phorate Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Phorate						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.33	6.89E+02	3.46E+04	1.99E-02	5.51	103
SA7	7.08	8.48E+02	4.27E+04	1.98E-02	5.49	77.6
SA6	17.8	2.69E+03	3.76E+04	7.14E-02	21.9	123
SA5	70.8	9.25E+03	4.10E+04	2.25E-01	70.8	100
SA4	178	2.55E+04	4.54E+04	5.61E-01	177	99.7
SA3	708	8.29E+04	3.80E+04	2.18E+00	693	97.9
SA2	1780	2.14E+05	3.92E+04	5.44E+00	1730	97.2
SA1	3550	4.57E+05	4.02E+04	1.14E+01	3610	102

a. Internal standard is dichlorvos d-6

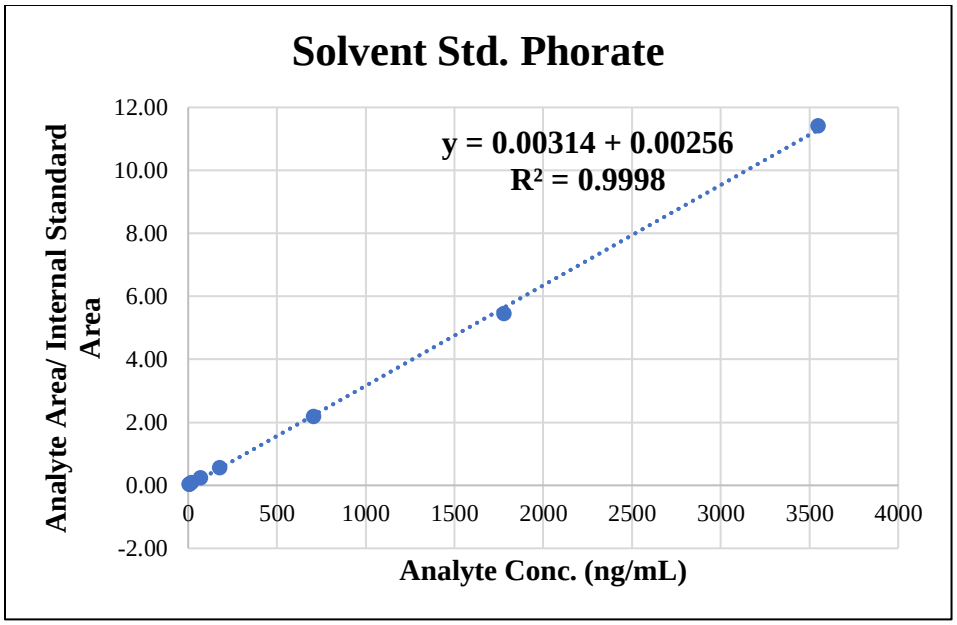

Figure A17: Solvent standards for phorate with a 1/x weighting

Table A22: Solvent Standards Terbufos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Terbufos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	3.35	6.43E+02	3.46E+04	1.86E-02	2.88	85.8
SA7	4.45	1.32E+03	4.27E+04	3.10E-02	5.12	115
SA6	11.1	2.29E+03	3.76E+04	6.08E-02	10.5	94.8
SA5	44.5	1.08E+04	4.10E+04	2.62E-01	47	106
SA4	111	2.74E+04	4.54E+04	6.04E-01	109	98.1
SA3	445	9.38E+04	3.80E+04	2.47E+00	446	100
SA2	1110	2.44E+05	3.92E+04	6.21E+00	1120	101
SA1	2230	4.92E+05	4.02E+04	1.22E+01	2220	99.4

a. Internal standard is dichlorvos d-6

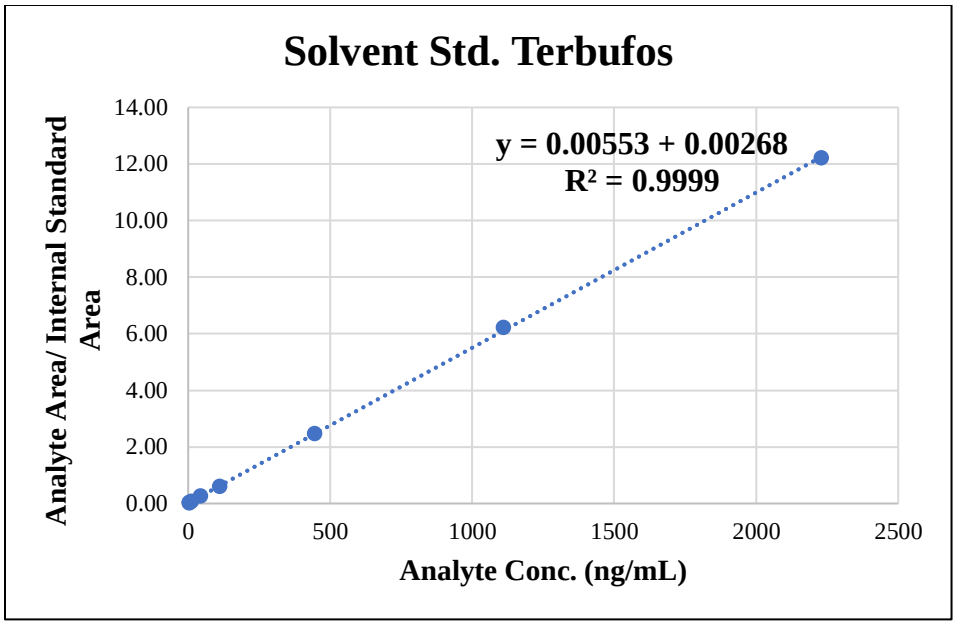

Figure A18: Solvent standards for terbufos with a 1/x weighting

Table A23: Solvent Standards Trichlorfon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Trichlorfon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	17.9	1.09E+03	3.46E+04	3.15E-02	16.1	90.1
SA7	23.9	1.72E+03	4.27E+04	4.01E-02	23.4	98
SA6	59.8	3.17E+03	3.76E+04	8.41E-02	60.5	101
SA5	239	1.24E+04	4.10E+04	3.02E-01	244	102
SA4	598	3.37E+04	4.54E+04	7.42E-01	615	103
SA3	2390	1.15E+05	3.80E+04	3.02E+00	2530	106
SA2	5980	2.84E+05	3.92E+04	7.24E+00	6100	102
SA1	11900	5.54E+05	4.02E+04	1.38E+01	11600	97.6

a. Internal standard is dichlorvos d-6

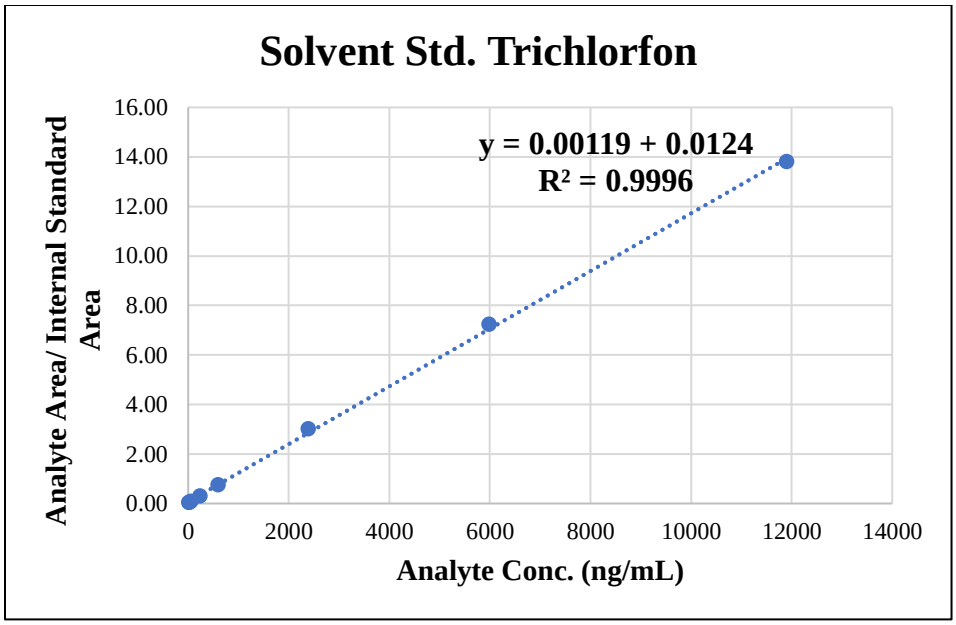

Figure A19: Solvent standards for trichlorfon with a 1/x weighting.

Table A24: Matrix Standards Chlorpyrifos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Chlorpyrifos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	19.1	1.16E+03	3.82E+04	3.03E-02	19.8	104
MA8-2	19.1	8.95E+02	3.66E+04	2.44E-02	14.6	76.5
MA8-3	19.1	1.05E+03	3.83E+04	2.74E-02	17.2	90.3
MA8-4	19.1	1.11E+06	4.15E+04	2.67E+01	23500	123000
MA8-5	19.1	6.18E+03	3.31E+04	1.87E-01	158	826
MA8-6	19.1	2.52E+03	5.37E+04	4.69E-02	34.4	180
MA7	25.5	1.53E+03	4.27E+04	3.58E-02	24.6	96.6
MA6	63.8	2.88E+03	3.61E+04	7.98E-02	63.4	99.3
MA5	255	1.38E+04	4.43E+04	3.12E-01	268	105
MA4	638	2.59E+02	3.40E+03	7.62E-02	60.2	9.44
MA3	2550	1.16E+05	4.11E+04	2.83E+00	2480	97.3
MA2	6380	2.59E+05	3.74E+04	6.91E+00	6080	95.3
MA1	12800	7.63E+05	5.10E+04	1.49E+01	13200	103

a. Internal standard is dichlorvos d-6

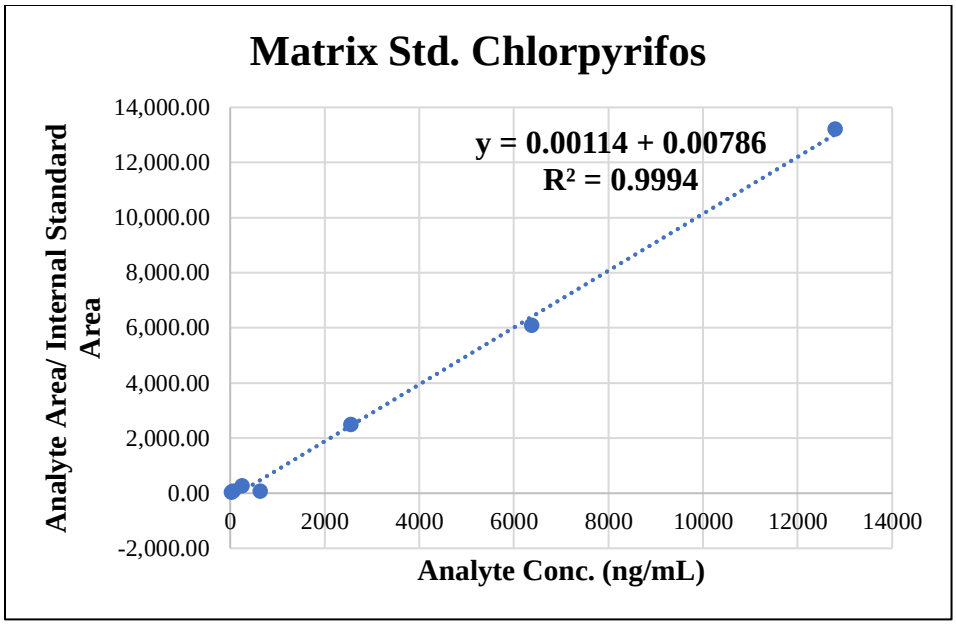

Figure A20: Matrix standards for chlorpyrifos with a 1/x weighting.

Table A25: Matrix Standards Coumaphos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Coumaphos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	5.18	9.31E+02	3.82E+04	2.44E-02	5.25	101
MA8-2	5.18	1.02E+03	3.66E+04	2.78E-02	6.26	121
MA8-3	5.18	1.04E+03	3.83E+04	2.72E-02	6.09	118
MA8-4	5.18	9.56E+02	4.15E+04	2.31E-02	4.86	93.9
MA8-5	5.18	7.93E+02	3.31E+04	2.40E-02	5.14	99.2
MA8-6	5.18	1.37E+03	5.37E+04	2.55E-02	5.59	108
MA7	6.9	1.44E+03	4.27E+04	3.36E-02	7.97	116
MA6	17.3	2.05E+03	3.61E+04	5.67E-02	14.8	85.3
MA5	69	1.08E+04	4.43E+04	2.43E-01	69.5	101
MA4	173	2.57E+02	3.40E+03	7.57E-02	20.3	11.8
MA3	690	9.54E+04	4.11E+04	2.32E+00	681	98.7
MA2	1730	2.12E+05	3.74E+04	5.67E+00	1670	96.3
MA1	3450	6.12E+05	5.10E+04	1.20E+01	3520	102

a. Internal standard is dichlorvos d-6

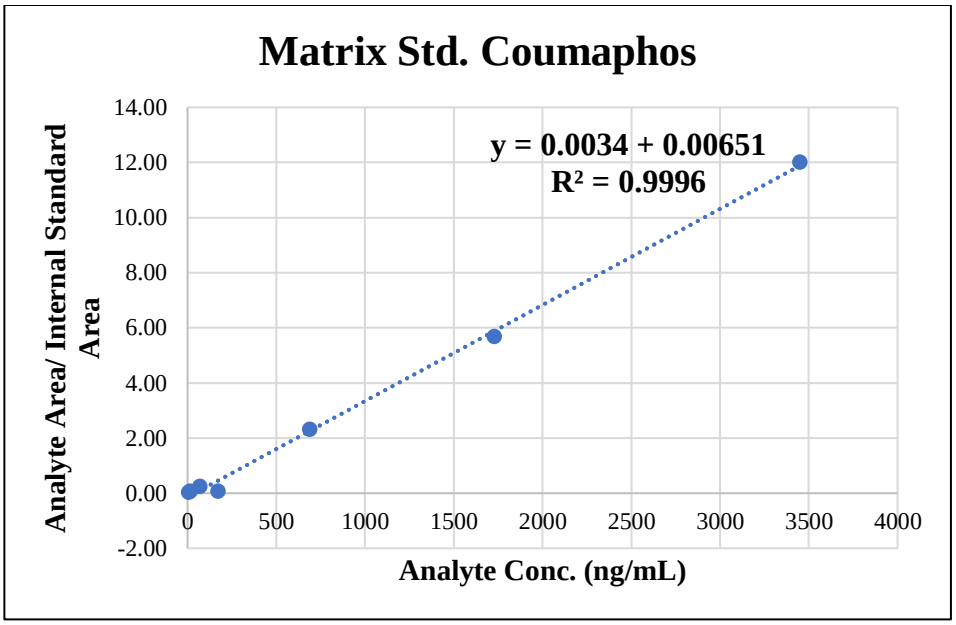

Figure A21: Matrix standards for coumaphos with a 1/x weighting.

Table A26: Matrix Standards Diazinon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Diazinon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	0.148	6.58E+02	3.82E+04	1.72E-02	0.138	93.5
MA8-2	0.148	7.86E+02	3.66E+04	2.15E-02	0.176	119
MA8-3	0.148	9.20E+02	3.83E+04	2.40E-02	0.198	134
MA8-4	0.148	9.90E+02	4.15E+04	2.39E-02	0.197	133
MA8-5	0.148	8.36E+02	3.31E+04	2.53E-02	0.209	141
MA8-6	0.148	1.28E+03	5.37E+04	2.38E-02	0.197	133
MA7	0.197	9.88E+02	4.27E+04	2.31E-02	0.19	96.5
MA6	0.493	2.12E+03	3.61E+04	5.88E-02	0.503	102
MA5	1.97	1.08E+04	4.43E+04	2.43E-01	2.12	108
MA4	4.93	6.26E+02	3.40E+03	1.84E-01	1.61	32.6
MA3	19.7	9.33E+04	4.11E+04	2.27E+00	19.9	101
MA2	49.3	2.07E+05	3.74E+04	5.54E+00	48.7	98.7
MA1	98.5	5.74E+05	5.10E+04	1.12E+01	98.8	100

a. Internal standard is dichlorvos d-6

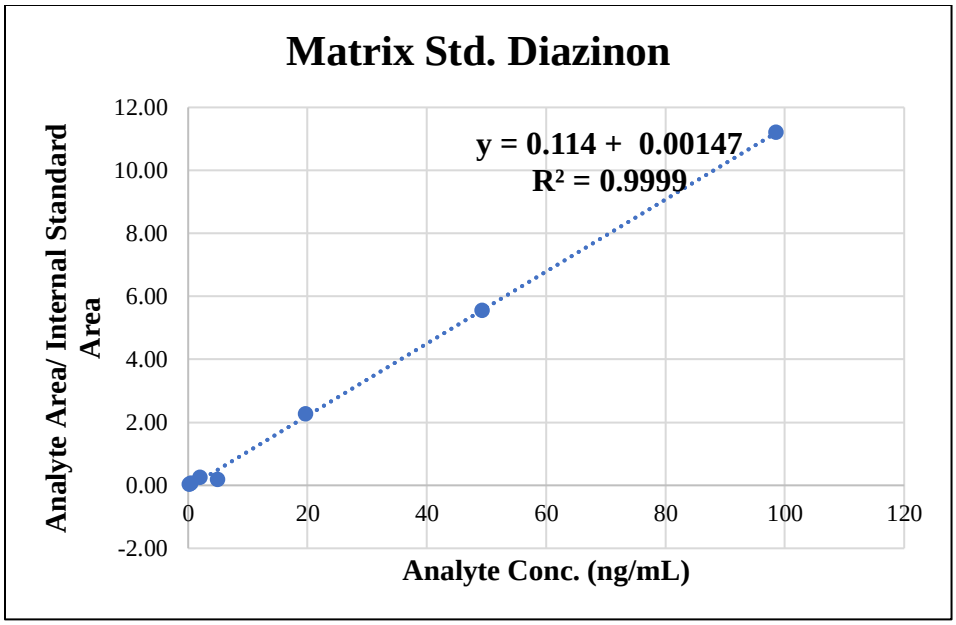

Figure A22: Matrix standards for diazinon with a 1/x weighting.

Table A27: Matrix Standards Dichlorvos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Dichlorvos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	5.15	1.33E+03	3.82E+04	3.47E-02	4.36	84.6
MA8-2	5.15	1.32E+03	3.66E+04	3.61E-02	4.68	90.9
MA8-3	5.15	1.29E+03	3.83E+04	3.38E-02	4.13	80.3
MA8-4	5.15	1.52E+03	4.15E+04	3.66E-02	4.81	93.4
MA8-5	5.15	1.20E+03	3.31E+04	3.62E-02	4.7	91.3
MA8-6	5.15	1.74E+03	5.37E+04	3.23E-02	3.77	73.3
MA7	6.85	1.77E+03	4.27E+04	4.14E-02	5.96	87.1
MA6	17.1	3.46E+03	3.61E+04	9.59E-02	19.2	112
MA5	68.5	1.42E+04	4.43E+04	3.22E-01	73.9	108
MA4	171	2.58E+03	3.40E+03	7.61E-01	180	106
MA3	685	1.19E+05	4.11E+04	2.91E+00	701	102
MA2	1710	2.72E+05	3.74E+04	7.27E+00	1760	103
MA1	3430	7.06E+05	5.10E+04	1.38E+01	3350	97.7

a. Internal standard is dichlorvos d-6

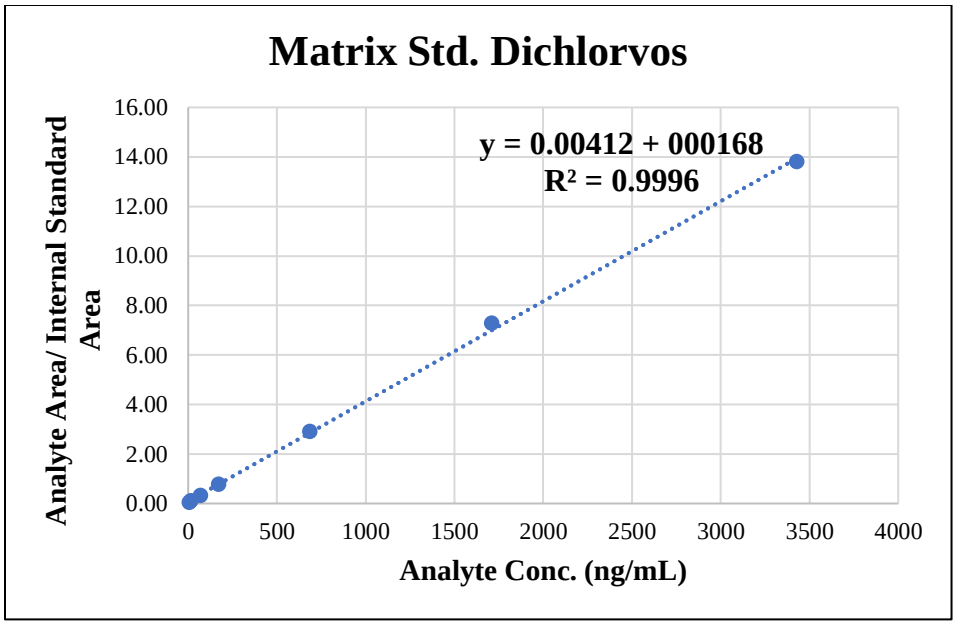

Figure A23: Matrix standards for dichlorvos with a 1/x weighting.

Table A28: Matrix Standards Malathion Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Malathion						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	2.2	8.62E+02	3.82E+04	2.26E-02	2.26	103
MA8-2	2.2	8.81E+02	3.66E+04	2.41E-02	2.45	111
MA8-3	2.2	9.81E+02	3.83E+04	2.56E-02	2.65	120
MA8-4	2.2	9.39E+02	4.15E+04	2.27E-02	2.27	103
MA8-5	2.2	6.62E+02	3.31E+04	2.00E-02	1.93	87.8
MA8-6	2.2	1.12E+03	5.37E+04	2.09E-02	2.04	92.7
MA7	2.93	1.20E+03	4.27E+04	2.81E-02	2.97	101
MA6	7.31	2.34E+03	3.61E+04	6.48E-02	7.64	104
MA5	29.3	9.80E+03	4.43E+04	2.21E-01	27.6	94.2
MA4	73.1	7.41E+02	3.40E+03	2.18E-01	27.2	37.2
MA3	293	9.27E+04	4.11E+04	2.26E+00	287	97.9
MA2	731	2.11E+05	3.74E+04	5.64E+00	718	98.3
MA1	1460	5.93E+05	5.10E+04	1.16E+01	1480	101

a. Internal standard is dichlorvos d-6

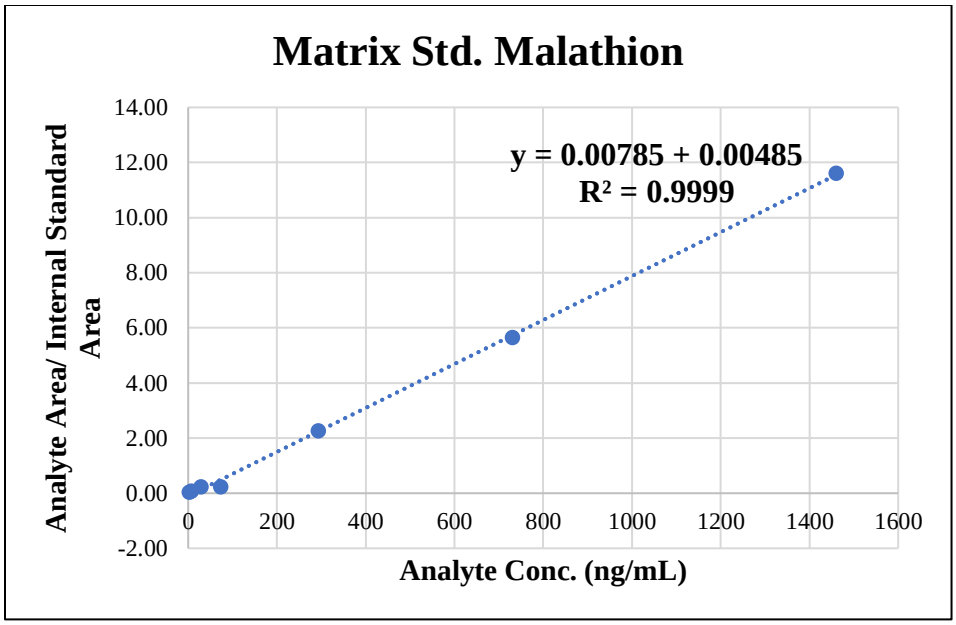

Figure A24: Matrix standards for malathion with a 1/x weighting.

Table A29: Matrix Standards Phorate Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Phorate						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	5.33	8.54E+02	3.82E+04	2.24E-02	5.64	106
MA8-2	5.33	7.74E+02	3.66E+04	2.11E-02	5.29	99.2
MA8-3	5.33	8.44E+02	3.83E+04	2.21E-02	5.55	104
MA8-4	5.33	8.51E+02	4.15E+04	2.05E-02	5.11	95.9
MA8-5	5.33	6.28E+02	3.31E+04	1.90E-02	4.67	87.6
MA8-6	5.33	1.18E+03	5.37E+04	2.20E-02	5.54	104
MA7	7.08	1.25E+03	4.27E+04	2.92E-02	7.58	107
MA6	17.8	2.22E+03	3.61E+04	6.16E-02	16.9	94.8
MA5	70.8	1.08E+04	4.43E+04	2.45E-01	69.3	97.8
MA4	178	2.99E+02	3.40E+03	8.80E-02	24.4	13.7
MA3	708	9.58E+04	4.11E+04	2.33E+00	667	94.2
MA2	1780	2.29E+05	3.74E+04	6.11E+00	1750	98.3
MA1	3550	6.46E+05	5.10E+04	1.27E+01	3620	102

a. Internal standard is dichlorvos d-6

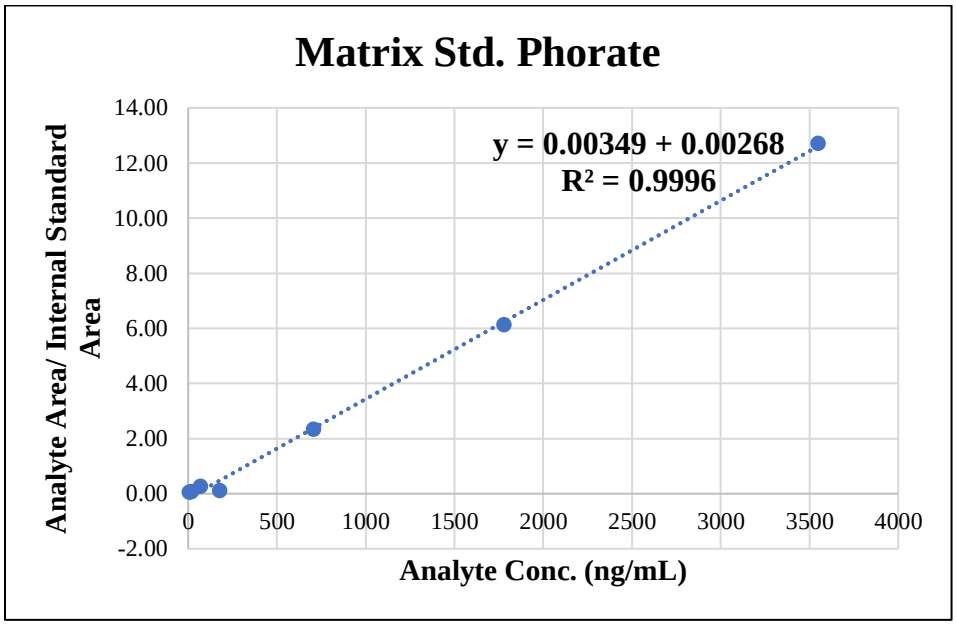

Figure A25: Matrix standards for phorate with a 1/x weighting.

Table A30: Matrix Standards Terbufos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Terbufos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	3.35	9.77E+02	3.82E+04	2.56E-02	4.1	122
MA8-2	3.35	7.20E+02	3.66E+04	1.97E-02	3.14	93.9
MA8-3	3.35	9.98E+02	3.83E+04	2.61E-02	4.19	125
MA8-4	3.35	9.71E+02	4.15E+04	2.34E-02	3.75	112
MA8-5	3.35	9.26E+02	3.31E+04	2.80E-02	4.49	134
MA8-6	3.35	1.45E+03	5.37E+04	2.71E-02	4.35	130
MA7	4.45	1.07E+03	4.27E+04	2.51E-02	4.03	90.5
MA6	11.1	2.52E+03	3.61E+04	6.97E-02	11.2	101
MA5	44.5	1.15E+04	4.43E+04	2.59E-01	41.8	93.9
MA4	111	2.11E+02	3.40E+03	6.23E-02	10	9.04
MA3	445	1.07E+05	4.11E+04	2.61E+00	421	94.7
MA2	1110	2.37E+05	3.74E+04	6.33E+00	1020	92.2
MA1	2230	7.39E+05	5.10E+04	1.45E+01	2340	105

a. Internal standard is dichlorvos d-6

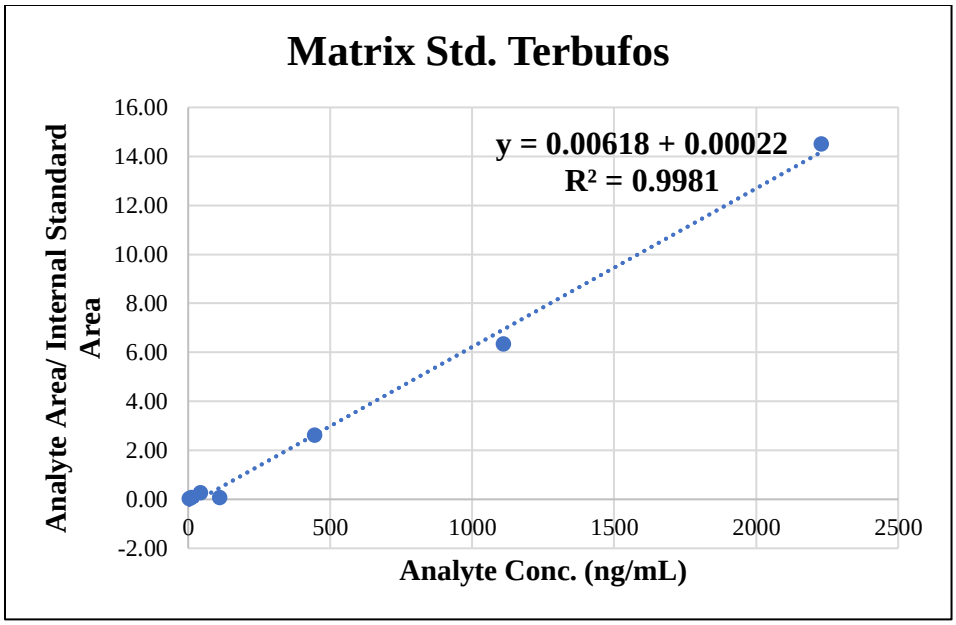

Figure A26: Matrix standards for terbufos with a 1/x weighting.

Table A31: Matrix Standards Trichlorfon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Trichlorfon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
MA8-1	17.9	1.05E+03	3.82E+04	2.74E-02	17.4	97
MA8-2	17.9	1.10E+03	3.66E+04	3.00E-02	19.6	109
MA8-3	17.9	9.16E+02	3.83E+04	2.39E-02	14.4	80.6
MA8-4	17.9	9.34E+02	4.15E+04	2.25E-02	13.2	73.9
MA8-5	17.9	9.14E+02	3.31E+04	2.76E-02	17.6	98.2
MA8-6	17.9	1.29E+03	5.37E+04	2.40E-02	14.5	80.7
MA7	23.9	1.48E+03	4.27E+04	3.47E-02	23.6	98.5
MA6	59.8	2.76E+03	3.61E+04	7.63E-02	58.9	98.5
MA5	239	1.33E+04	4.43E+04	3.00E-01	249	104
MA4	598	3.08E+03	3.40E+03	9.07E-01	765	128
MA3	2390	1.14E+05	4.11E+04	2.77E+00	2340	98.1
MA2	5980	2.81E+05	3.74E+04	7.50E+00	6370	106
MA1	11900	6.94E+05	5.10E+04	1.36E+01	11500	97.1

a. Internal standard is dichlorvos d-6

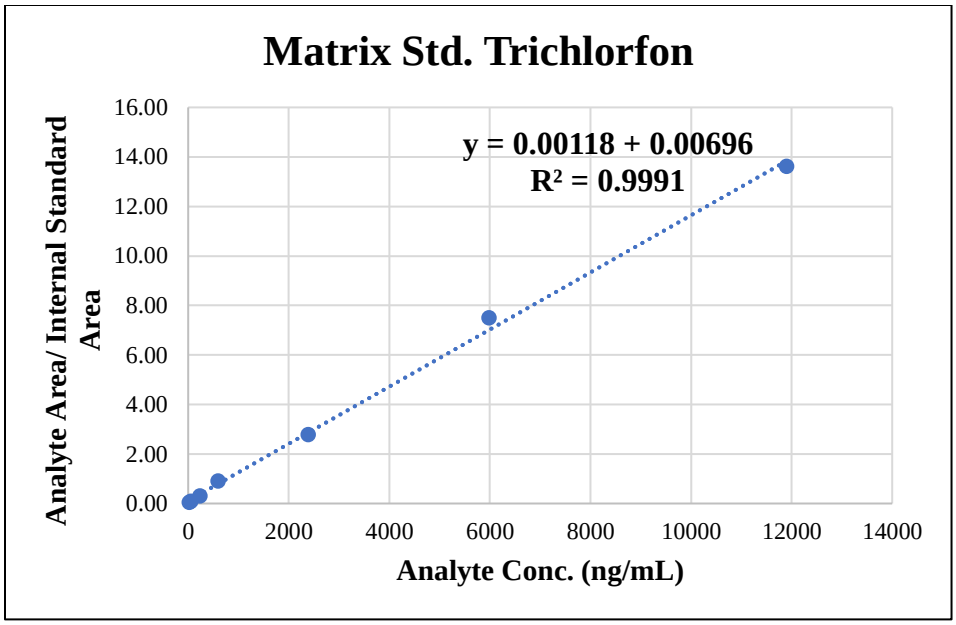

Figure A27: Matrix standards for trichlorfon with a 1/x weighting.

Appendix B: Application of Validated Method

Table B32: Solvent Standards Concentrations by Concentration Factors (ng/mL)

OP	500x	250x	100x	25x	10x	2.5x	x	0.75x
Chlorpyrifos	12800	6410	2560	641	256	64.1	25.6	19.2
Coumaphos	3410	1710	683	171	68.3	17.1	6.83	5.12
Diazinon	110	54.9	22.0	5.49	2.20	0.549	0.220	0.165
Dichlorvos	3850	1930	770	193	77.0	19.3	7.70	5.78
Malathion	1640	819	328	81.9	32.8	8.19	3.28	2.46
Phorate	3740	1870	748	187	74.8	18.7	7.48	5.61
Terbufos	2600	1300	520	130	52.0	13.0	5.20	3.90
Trichlorfon	12100	6030	2410	603	241	60.3	24.1	18.1

Table B33: Solvent Standards Chlorpyrifos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Chlorpyrifos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SStd8	19.2	3.59E+03	3.06E+05	1.17E-02	2.78	14.5
SStd7	25.6	7.37E+03	3.38E+05	2.18E-02	22.1	86.1
SStd6	64.1	1.45E+04	2.69E+05	5.40E-02	84.1	131
SStd5	256	4.19E+04	2.51E+05	1.67E-01	301	118
SStd4	641	1.57E+05	3.12E+05	5.05E-01	950	148
SStd3	2560	4.14E+05	2.85E+05	1.45E+00	2770	108
SStd2	6410	1.01E+06	3.12E+05	3.25E+00	6220	97.1
SStd1	12800	2.07E+06	3.19E+05	6.48E+00	12400	97.1

a. Internal standard is dichlorvos d-6

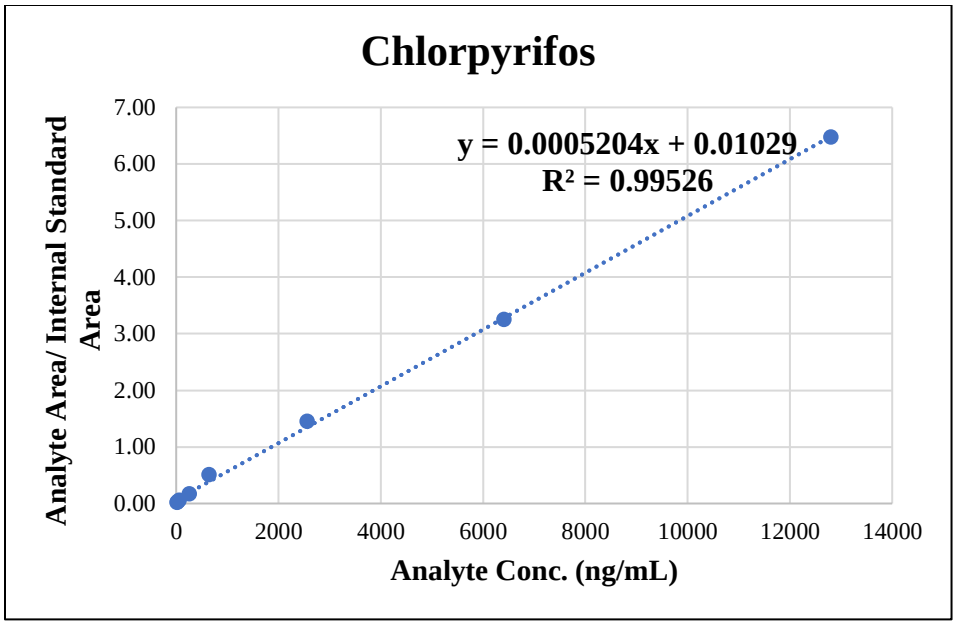

Figure B28: Solvent standards for chlorpyrifos with a 1/x weighting.

Table B34: Solvent Standards Coumaphos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Coumaphos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.12	4.80E+03	3.06E+05	1.57E-02	3.35	65.5
SA7	6.83	9.85E+03	3.38E+05	2.91E-02	7.14	105
SA6	17.1	2.05E+04	2.69E+05	7.62E-02	20.4	120
SA5	68.3	6.56E+04	2.51E+05	2.62E-01	72.8	107
SA4	171	2.34E+05	3.12E+05	7.50E-01	211	123
SA3	683	5.51E+05	2.85E+05	1.93E+00	544	79.7
SA2	1710	1.83E+06	3.12E+05	5.86E+00	1650	96.7
SA1	3410	4.03E+06	3.19E+05	1.26E+01	3560	104

a. Internal standard is dichlorvos d-6

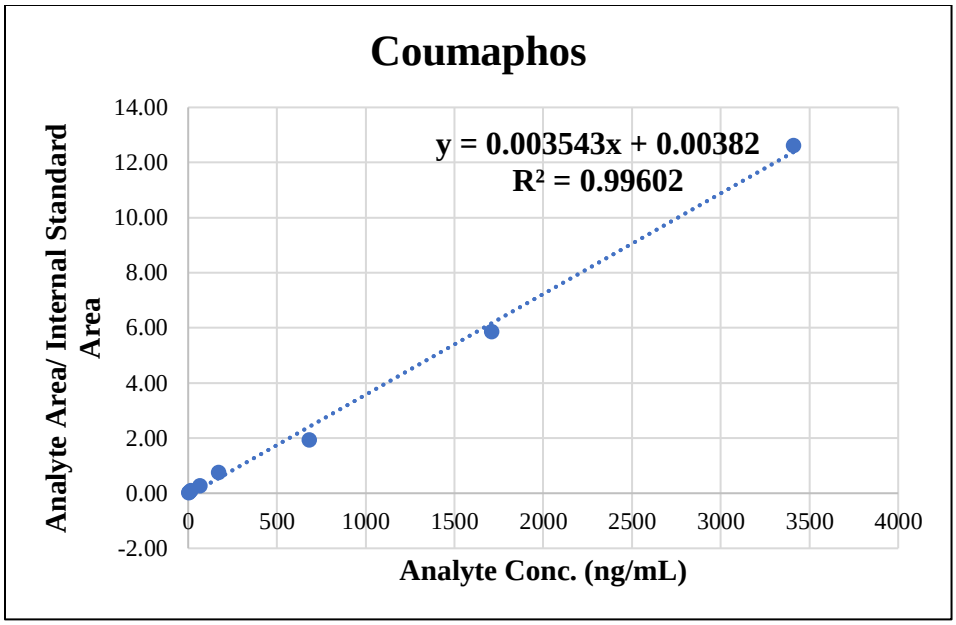

Figure B29: Solvent standards for coumaphos with a 1/x weighting.

Table B35: Solvent Standards Diazinon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Diazinon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	0.165	1.52E+03	3.06E+05	4.95E-03	0.0988	59.9
SA7	0.22	2.23E+03	3.38E+05	6.60E-03	0.207	93.9
SA6	0.549	3.66E+03	2.69E+05	1.36E-02	0.666	121
SA5	2.2	1.08E+04	2.51E+05	4.30E-02	2.59	118
SA4	5.49	3.49E+04	3.12E+05	1.12E-01	7.09	129
SA3	22	7.79E+04	2.85E+05	2.73E-01	17.7	80.3
SA2	54.9	2.41E+05	3.12E+05	7.71E-01	50.2	91.5
SA1	110	5.72E+05	3.19E+05	1.79E+00	117	106

a. Internal standard is dichlorvos d-6

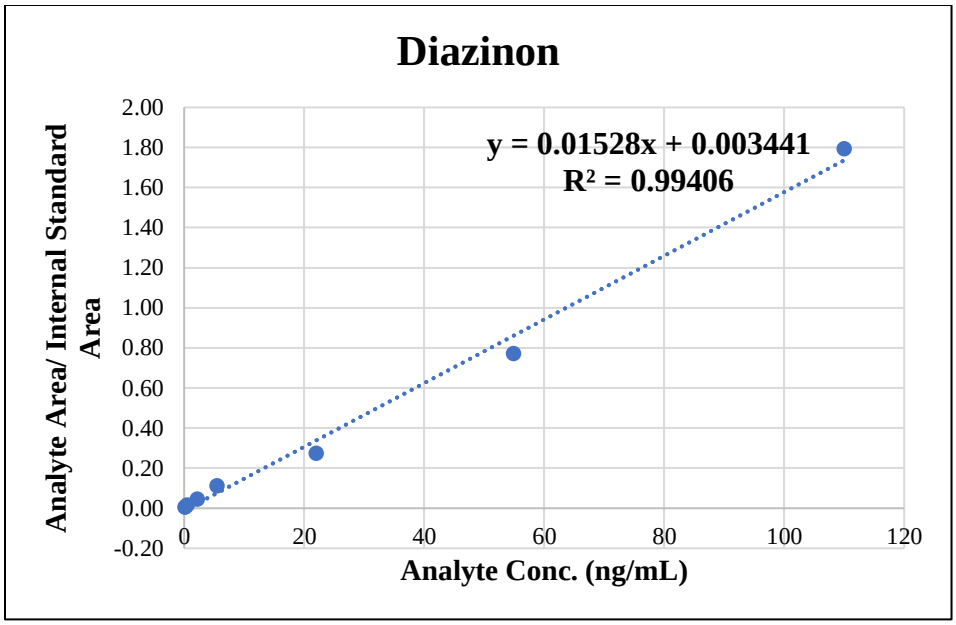

Figure B30: Solvent standards for diazinon with a 1/x weighting.

Table B36: Solvent Standards Dichlorvos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Dichlorvos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.78	7.78E+03	3.06E+05	2.54E-02	< 0	N/A
SA7	7.7	1.08E+04	3.38E+05	3.20E-02	< 0	N/A
SA6	19.3	2.55E+04	2.69E+05	9.50E-02	50.6	262
SA5	77	6.14E+04	2.51E+05	2.45E-01	180	233
SA4	193	1.67E+05	3.12E+05	5.37E-01	431	223
SA3	770	2.75E+05	2.85E+05	9.65E-01	800	104
SA2	1930	6.38E+05	3.12E+05	2.04E+00	1730	89.5
SA1	3850	1.37E+06	3.19E+05	4.31E+00	3680	95.5

a. Internal standard is dichlorvos d-6

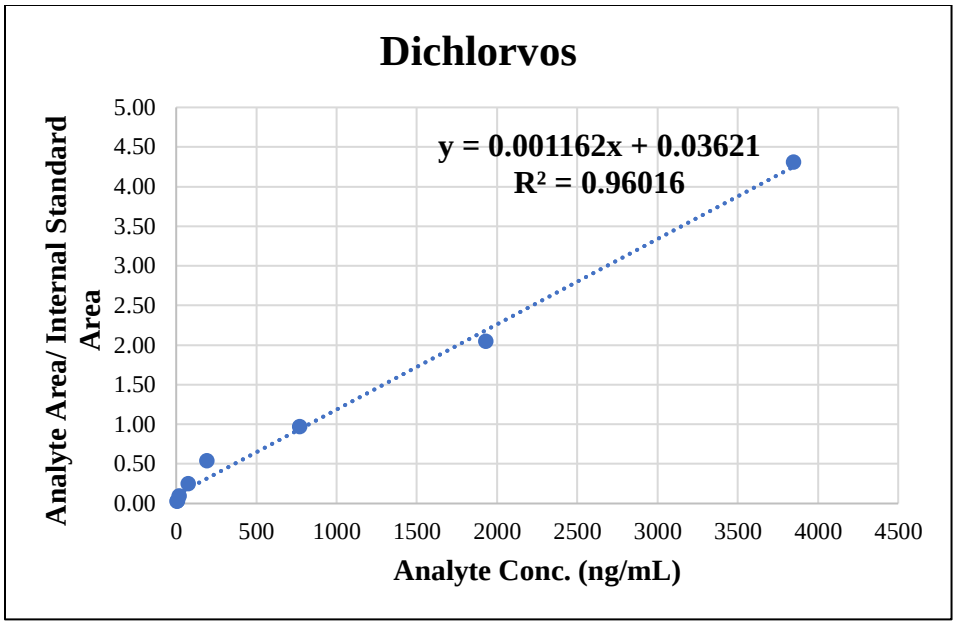

Figure B31: Solvent standards for dichlorvos with a 1/x weighting.

Table B37: Solvent Standards Malathion Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Malathion						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	2.46	8.67E+03	3.06E+05	2.83E-02	1.6	65.2
SA7	3.28	1.18E+04	3.38E+05	3.48E-02	3.16	96.4
SA6	8.19	1.69E+04	2.69E+05	6.30E-02	9.94	121
SA5	32.8	4.10E+04	2.51E+05	1.63E-01	34.2	104
SA4	81.9	1.47E+05	3.12E+05	4.72E-01	108	132
SA3	328	3.18E+05	2.85E+05	1.11E+00	263	80.3
SA2	819	1.02E+06	3.12E+05	3.28E+00	785	95.8
SA1	1640	2.27E+06	3.19E+05	7.12E+00	1710	104

a. Internal standard is dichlorvos d-6

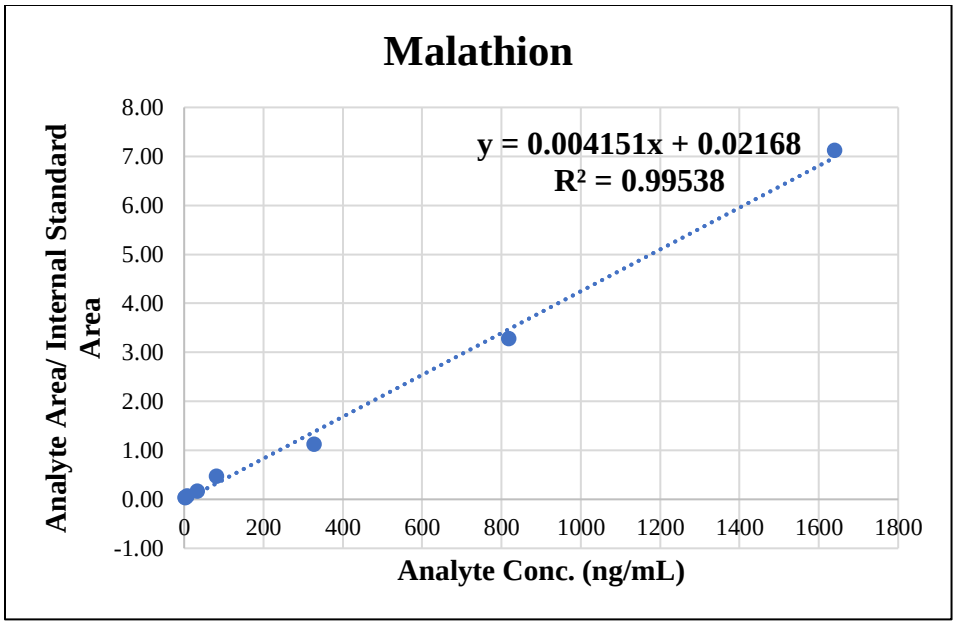

Figure B32: Solvent standards for malathion with a 1/x weighting.

Table B38: Solvent Standards Phorate Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Phorate						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	5.61	8.25E+02	3.06E+05	2.70E-03	0.99	17.6
SA7	7.48	2.15E+03	3.38E+05	6.36E-03	8.72	117
SA6	18.7	3.87E+03	2.69E+05	1.44E-02	25.7	138
SA5	74.8	1.07E+04	2.51E+05	4.25E-02	85.1	114
SA4	187	3.68E+04	3.12E+05	1.18E-01	245	131
SA3	748	9.12E+04	2.85E+05	3.20E-01	671	89.7
SA2	1870	2.42E+05	3.12E+05	7.77E-01	1640	87.6
SA1	3740	6.02E+05	3.19E+05	1.89E+00	3980	106

a. Internal standard is dichlorvos d-6

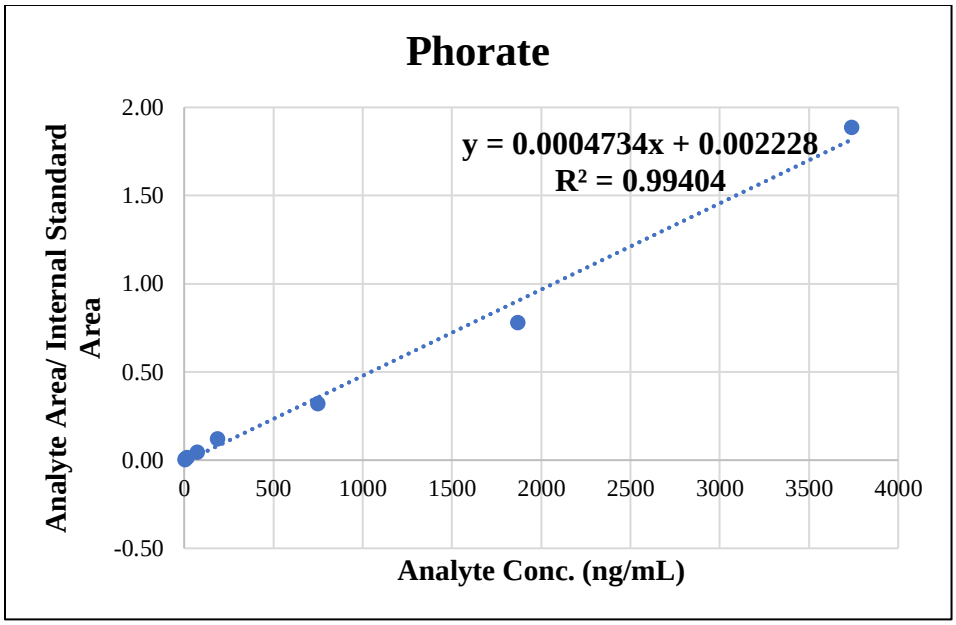

Figure B33: Solvent standards for phorate with a 1/x weighting.

Table B39: Solvent Standards Terbufos Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Terbufos						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts) [a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	3.9	2.66E+03	3.06E+05	8.68E-03	2.6	66.8
SA7	5.2	3.99E+03	3.38E+05	1.18E-02	4.96	95.4
SA6	13	7.11E+03	2.69E+05	2.65E-02	16.1	124
SA5	52	2.20E+04	2.51E+05	8.76E-02	62.3	120
SA4	130	6.06E+04	3.12E+05	1.94E-01	143	110
SA3	520	1.69E+05	2.85E+05	5.93E-01	445	85.6
SA2	1300	5.03E+05	3.12E+05	1.61E+00	1220	93.6
SA1	2600	1.15E+06	3.19E+05	3.61E+00	2730	105

a. Internal standard is dichlorvos d-6

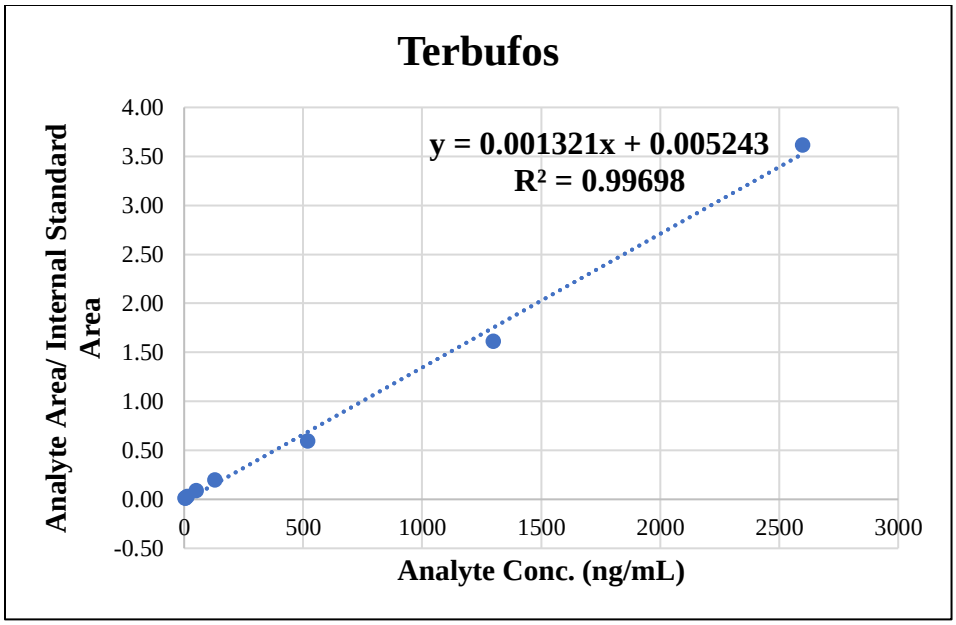

Figure B34: Solvent standards for terbufos with a 1/x weighting.

Table B40: Solvent Standards Trichlorfon Analyte Concentrations (ng/mL), Area Ratio, Recovered Analyte Concentrations (ng/mL), and % Accuracy.

Trichlorfon						
Sample Name	Analyte Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts)[a]	Area Ratio	Recovered Analyte Concentration (ng/mL)	Accuracy (%)
SA8	18.1	0.00E+00	3.06E+05	0.00E+00	No Peak	0
SA7	24.1	0.00E+00	3.38E+05	0.00E+00	No Peak	0
SA6	60.3	3.19E+03	2.69E+05	1.19E-02	87.8	146
SA5	241	1.38E+04	2.51E+05	5.52E-02	184	76.2
SA4	603	7.41E+04	3.12E+05	2.38E-01	588	97.5
SA3	2410	2.45E+05	2.85E+05	8.61E-01	1970	81.6
SA2	6030	7.59E+05	3.12E+05	2.43E+00	5450	90.3
SA1	12100	1.89E+06	3.19E+05	5.92E+00	13200	109

a. Internal standard is dichlorvos d-6

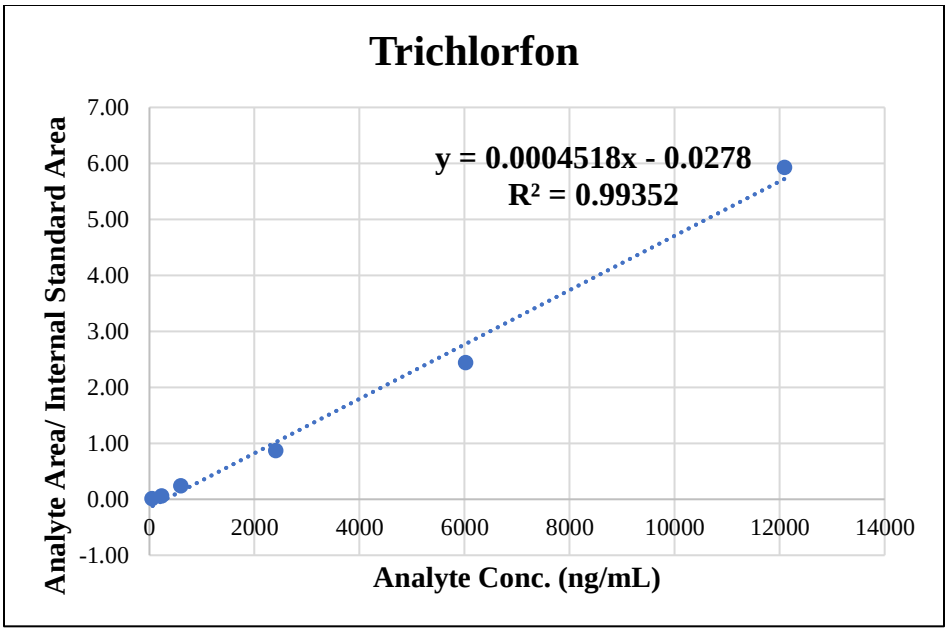

Figure B35: Solvent standards for trichlorfon with a 1/x weighting.

Appendix C: Chromatograms

The chromatograms are attached as a supplemental file.

Appendix D: Instrumental Parameters

Instrumental Parameters are attached as a supplemental file.

Appendix E: IRB Approval Letters

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-70 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · **www.ecu.edu/irb**

Notification of Initial Approval: Expedited

From: Biomedical IRB
To: Anne Marie Spuches
CC:

Date: 9/14/2015
Re: UMCIRB 15-000984
"The association of organophosphates and heavy metals with obesity and the metabolic syndrome: a study of patients in eastern North Carolina

I am pleased to inform you that your Expedited Application was approved. Approval of the study and any consent form(s) is for the period of 9/13/2015 to 9/12/2016. The research study is eligible for review under expedited category # 2,7. The Chairperson (or designee) deemed this study no more than minimal risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The Investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Name	Description
Grant application	Study Protocol or Grant Application
HIPAA-Authorization-OP Study_2015.docx	HIPAA Authorization
OP and HM consent 5-28-2015.doc	Consent Forms
OP and HM Survey 6-16-15	Surveys and Questionnaires
Reviews-Preparatory-to-Research-Form AMS.doc	HIPAA Authorization

The Chairperson (or designee) does not have a potential for conflict of interest on this study.

IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-70 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · www.ecu.edu/irb

Notification of Continuing Review Approval: Expedited

From: Biomedical IRB
To: Anne Marie Spuches
CC:

Date: 8/25/2016
Re: CR00004872
UMCIRB 15-000984
"The association of organophosphates and heavy metals with obesity and the metabolic syndrome: a study of patients in eastern North Carolina

The continuing review of your expedited study was approved. Approval of the study and any consent form(s) is for the period of 8/25/2016 to 8/24/2017. This research study is eligible for review under expedited category # 2,7. The Chairperson (or designee) deemed this study no more than minimal risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The Investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document	Description
Grant application(0.01)	Study Protocol or Grant Application
HIPAA-Authorization-for-Future-Research-1-11-16.docx(0.02)	HIPAA Authorization
HIPAA-Authorization-OP Study_2015.docx(0.04)	HIPAA Authorization
OP and HM consent 5-28-2015.doc(0.11)	Consent Forms
OP and HM Survey 6-16-15(0.01)	Surveys and Questionnaires
Reviews-Preparatory-to-Research-Form AMS.doc(0.01)	HIPAA Authorization

The Chairperson (or designee) does not have a potential for conflict of interest on this study.

IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418