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Kimberly D. Smith

Analytical Methods for Pesticides and their Degradation Products in Soil

By

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An Abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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Abstract

The pesticide industry specifically has made a remarkable impact in the protection of food supplies but has also created unanticipated environmental and human health adverse effects. Evidence has linked pesticide exposure to almost every type of cancer possible in addition to neurodegenerative diseases, newborn deficiencies and endocrine disruption. Despite this insurmountable evidence against pesticides, the pesticide industry has become so integrated into our society that many believe that reconsidering or abolishing the industry would have multiple economic consequences (Rosenbaum 1998). Inevitably, the industry is here to stay for the time being and it becomes the duty of public health officials and scientists to limit pesticide exposure and educate the public in awareness of toxic side effects to their health and the environment.

A critical part in identifying the presence of pesticides, their toxicity and ultimately their possible effect on people and the environment is the determination of the exact amount of pesticide that is present. Analytical methods are utilized to provide quantitative data to help answer many of these questions. Multiple analytical methods were examined in this research for their utility to analyze and quantitate pesticides and their degradation products in soil. Traditional and cartridge-assisted liquid-liquid extraction, solid-phase extraction including C18, polymeric, ion-exchange and a recent innovation: molecular imprinted polymer, and accelerated solvent extraction are evaluated for extraction of the target analytes. Gas chromatographic and liquid chromatographic techniques are also investigated to determine optimal instrumental analysis. All methods were assessed for analytical parameters typical in method development: accuracy, precision, sensitivity, etc.

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List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

List of Tables

Table 3.20 Linearity of Target Analytes with MISPE

List of Acronyms

3-PBA	3-Phenoxybenzoic Acid
4-F3PBA	4-Fluoro-3-Phenoxybenzoic Acid
4-NP	4-Nitrophenol (PNP also used)
AA	Ammonium Acetate
AED	Atomic Emission Detection
AF	Ammonium Formate
API	Atmospheric Pressure Ionization
ASE	Accelerated Solvent Extraction (PLE also used)
CDC	US Centers for Disease Control
CDCA	Chrysanthemum Dicarboxylic Acid
CE	Collision Energy
CFCA	3, 3, 3-Trifluoroprop-1-Enyl-2, 2 Dimethylcyclopropane Carboxylic Acid
CI	Chemical Ionization
CID	Collision Induced Dissociation
CRM	Charged Residue Model
DAP	Dialkylphosphate
DBCA	Cis-3-(2, 2 Dibromovinyl) -2, 2 Dimethylcyclopropane-1-Carboxylic Acid
DCCA	3-(2, 2 Dichlorovinyl)-2, 2Dimethylcyclopropane Carboxylic Acid
DDD	Dichloro-Dipheny-Dichloroethane
DDE	Dichloro-Diphenyl-Dichloroethylene
DDT	Dichloro-Diphenyl-Trichloroethane
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DEDTP	Diethyldithiophosphate
DMP	Dimethylphosphate
DMTP	Dimethylthiophosphate
DMDTP	Dimethyldithiophosphate
ECD	Electron Capture Detection
EDTA	Ethylene Diamine Tetracetic Acid
EI	Electron Ionization
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionization
FQPA	Food Quality Protection Act
GC	Gas Chromatography
HESI	Heated Electrospray Ionization
HILIC	Hydrophilic Interaction Liquid Chromatography
IDL	Instrument Detection Limit
IE	Ion Exchange
IEM	Ion Evaporation Model
IMPY	1-Isopropyl-6-Methyl-4-Pyrimidol
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Detection Limit
LOG D	Distribution Coefficient

LOG P	Partition Coefficient
LOQ	Limit of Quantification
MCP	Monocrotophos
MDA	Malathion Dicarboxylic Acid
MDL	Method Detection Limit
MEOH	Methanol
MIP	Molecular Imprinted Polymer
MISPE	Molecular Imprinted Polymer Solid Phase Extraction
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry
NHANES	US National Health and Nutrition Examination Survey
NP	Normal Phase
NPD	Nitrogen Phosphorus Detection
OC	Organochlorine Pesticides
OP	Organophosphate Pesticides
PLE	Pressurized Liquid Extraction (ASE also used)
PNP	Paranitrophenol (4-NP also used)
PPB	Parts-Per-Billion
PPM	Parts-Per-Million
PPT	Parts-Per-Trillion
Q1-Q3	Quadrupole 1-3 of Mass Spectrometer
QC	Quality Control or Quality Characterization
RP	Reverse Phase
RSD	Relative Standard Deviation
SIM	Single Ion Monitoring
SFE	Supercritical Fluid Extraction
SN	Signal to Noise Ratio
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
SRM	Selected Reaction Monitoring
TCPY	3, 5, 6-Trichloro-2-Pyridinol
TEA	Triethylamine
TIC	Total Ion Current (Total Ion Chromatogram also used)

Table of Contents

CHAPTER 1: INTRODUCTION AND BACKGROUND

Chemical Industry

The chemical industry plays a constant, dynamic, controversial role in our society. Considered to be the first high-technology industry, chemicals have affected all sectors of society since the mid-19th century (Buccini 2004). The chemical industry converts raw materials into commodities that have extensive implications from the fairly innocuous soda industry to the more power-wielding weapon industry. As of 2004, the chemical industry, "… employs more than 10 million people worldwide, accounts of 7% of global income, 9% of international trade and an estimated $1.5 trillion in sales in 1998. …" (Buccini 2004). Viewed today as essential to our way of life, the realization of the negative ramifications of widespread usage of chemicals has grown alongside chemical industry growth. Scientists and the public alike are beginning to understand the adverse health and environmental impacts as a result of our dependency on chemical products. In 1959, a speaker for at the North American Wildlife Conference declared that "the conservation conscience" would never be a priority of the American people; however- three decades later, approximately 80% of Americans have supported environmentalist goals and environmental sustainability (Sale 1993). Over the years, this founded awareness has led to initiatives on chemicals, agreements and global partnerships like United Nations Environmental Program (UNEP), World Health Organization's Intergovernmental Forum on Chemical Safety (IFCS), World Wildlife Fund, EarthWatch and Greenpeace, etc. that has produced thousands of data and risk assessments on contemporary chemicals in an effort to promote environmental sustainability. This environmental movement or "green revolution" has become such a major cultural

phenomenon that has not held such weighty implications and provocative contention since the movement against slavery in the nineteenth century (Sale 1993).

Despite the constant barrage of information regarding chemical toxicities, there is still much that scientists still do not know. One of the difficulties in understanding chemical exposure is the inherent properties unique to each chemical. Furthermore, each chemical may have diverse behaviors and/or toxic actions dependent on the media in which it exists, be it in a factory, in the environment, or in our homes. Each living creature is exposed to multiple chemicals each day and moreover people and other living entities have varying susceptibilities and responses to chemicals based on their own genetic predisposition and life stage which further complicates discerning exposure-outcome relationships. Each chemical is said to have a "life cycle" (Bollinger 1997; Buccini 2004). The life cycle of a chemical is dependent on where it was manufactured, what it will be used for and how will it be disposed (Buccini 2004). The critical question is: how and to what extent are people and the environment exposed to the chemical at each point of the chemical's life cycle? This question is at the center of many debates over the last half of the century.

The roots of the controversy began post-World War II when technological change was heavily influenced to "….make mass production more efficient" (Ashford 1996). A streamlined approach to the workforce seemingly created a more prosperous and productive society in the 1950s and 1960s substituting many natural products for synthetic chemicals: plastics for paper, wood and metals; detergents for soaps; nitrogen

fertilizer for soil; pesticides for insect's natural predators as specific examples (Ashford 1996). Unfortunately, the benefits of the technological advancements made in society during these times belied a darker side that arose shortly thereafter. It has been estimated that half of applied fertilizers are washed away from fields to rivers that has led to an assortment of problems (Nosengo 2003). These problems are a direct result of overabundance of nitrates used in the fertilizers and have been implicated in the contamination of water resources and killing of various aquatic life, acidic rain and soils, deforestation, smog and global warming (Nosengo 2003). Another issue that erupted in the early 1970s is the use of phosphate-laden detergents as a replacement for soap for laundry cleaning. Detergents that have phosphate additives were found to reduce residual scum and particulates while aiding in killing of germs that otherwise remained with non-phosphate containing detergents (Knud-Hansen 1994). Despite the advantages of phosphate-containing detergents, excessive phosphates have accumulated in natural water resources leading to eutrophication and disruption of the natural ecosystem (Hammond 1971). Another source of conflict is due to our heavy reliance on pesticides and the toxic residues (parent pesticides and/or pesticide degradation products) left behind after application that remain in the environment or enter the food chain.

Pesticide Industry

After the release of *Silent Spring* in 1962 by Rachel Carson (Carson 1962) concern over the use of the insecticide DDT spread dramatically prompting restriction of its use in 1969 and complete ban in the US and other countries in 1972 (EPA 1972). Despite being banned for over 30 years, it still remains the focus of a geopolitical debate because it is still used in other countries albeit developing, malaria-endemic countries. Additionally, DDT and its breakdown products, DDE and DDD, have prolonged persistence in the environment and in people with residues still being found in the population (Gladen, Klebanoff et al. 2004). The third installment of the US Centers for Disease Control and Prevention (CDC) National Report on Human Exposure to Environmental Chemicals in 2005 reported that half of the tested population still had DDT and subsequent metabolites in their blood (CDC/NCEH 2005). The dilemma is the long-term, bioaccumulative property of DDT that not only affects human and wildlife health but the environmental as well. DDT and its breakdown products have been shown to have estrogenic and antiandrogenic properties as well as carcinogenic, reproductive and neurological and gestational side effects (Kupfer 1975; Garabrant, Held et al. 1992; Kelce, Stone et al. 1995; Chen, Hurd et al. 1997; Soliman, Smith et al. 1997; Eriksson and Talts 2000; Gray, Hotchkiss et al. 2001; Longnecker, Klebanoff et al. 2001; Snedeker 2001; Hardell, Lindstrom et al. 2002; Longnecker, Klebanoff et al. 2002). Additionally, the concept of chemical release and global distribution over time known as the “grasshopper effect”, only adds fire to the ongoing debate (Semeena and Lammel 2005).

Because of the controversy surrounding DDT use and subsequent ban, several classes of less environmentally persistent but potentially more acutely toxic pesticides have replaced organochlorines. The organophosphate (OP) class of pesticides is one such class. Organophosphates account for approximately 70% of all insecticides used in the United States across agricultural and non-agricultural markets since the early 1980's (EPA 2004). More recently, the pyrethroids class of pesticides has become a common replacement for both organochlorines (OCs) and organophosphates. Recently, the CDC reported pyrethroid insecticide exposure for the first time and concluded that the majority of the US population (age 6-59 years) was exposed to pyrethroid insecticides as indicated by the detection of a predominant urinary metabolite (3-phenxybenzoic acid) (CDC/NCEH 2005). Similar to DDT and other organochlorines pesticides, emerging studies suggesting adverse health outcomes associated with exposure grew parallel to the wide-spread use and popularity of these newer pesticides. EPA began phasing out chlorpyrifos and diazinon, two widely used organophosphate insecticides, in 2000, due to several studies finding a link between children and adverse health outcomes in relation to the usage of these pesticides (EPA 2000; EPA 2000; Cox, Kolb et al. 2005; Babu, Malik et al. 2006; De Silva, Samarawickrema et al. 2006; Holland, Furlong et al. 2006; Mense, Sengupta et al. 2006; Handal, Lozoff et al. 2007). Two studies have found links between smaller newborns (weight, height, head circumference) and use of these pesticides during pregnancy (Perera, Rauh et al. 2003; Whyatt, Rauh et al. 2004).

Adding to the increasing evidence of adverse health outcomes due to pesticide exposure is the reality that use of pesticide formulations is escalating as well. Increases in

production and expenditures of pesticides worldwide have been documented; Figure 1.1 below describes this trend from the last 60 years.

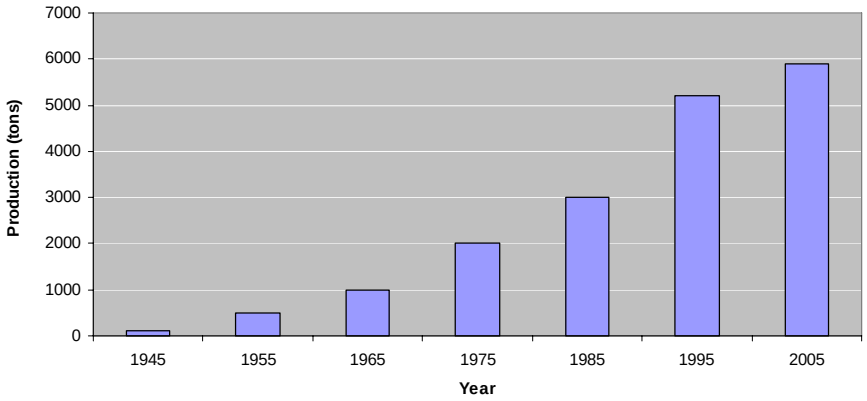


Figure 1.1 World production of formulated pesticides *Adapted from* (Agrochemical_Service 2000; Carvalho 2006).

The US has shown comparable increases in pesticide production, albeit at about one quarter of world production (EPA 2004). From 1945 to 1989, insecticide usage in the US has increased 10-fold (Horrigan, Lawrence et al. 2002). EPA has estimated that close to 900 million pounds of pesticide active ingredients were used across all sectors in 2001 as shown in Figure 1.2 (EPA 2004).

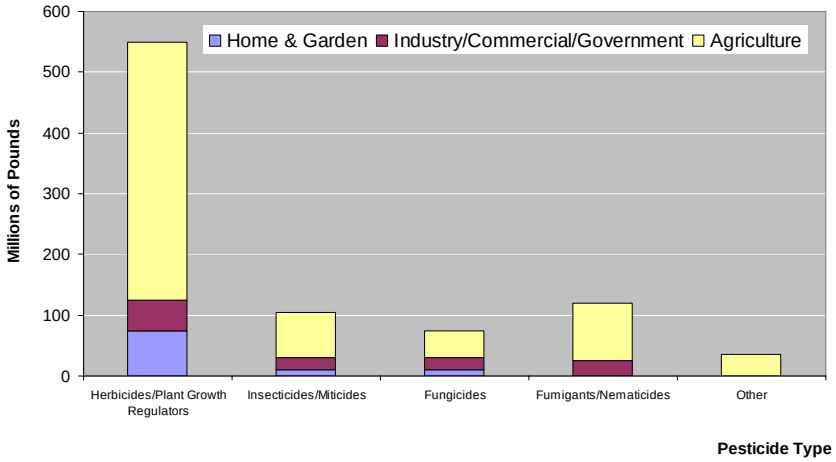


Figure 1.2 Amounts of Conventional Pesticide Active Ingredient used in the US by Pesticide Type and Market Sector in 2001 *Adapted from* (EPA 2004).

The pesticide industry has become so integrated into our agricultural production that many farmers believe that productivity cannot survive without them (Rosenbaum 1998). Unfortunately, high-volume pesticide application has the potentiality of affecting much more than the intended target. It has been estimated that only 0.1% of applied pesticide actually reaches the target pests, (Horrigan, Lawrence et al. 2002). There is an environmental and economic impact of excessive pesticide application that can be just as harmful and augment the pre-existing human health concerns. It has been estimated that honeybee colonies have dropped in the US from 4.4 million in 1985 to <1.9 million in 1997 due to indirect and direct effects from pesticides (Horrigan, Lawrence et al. 2002). Honeybees are involved in the pollination of an estimated $14 billion worth of US seeds and crops (Barrionuevo 2007). Another problem is the burgeoning resistance of many target insects from insecticides (<20 in 1950 to >500 in 1990) and plants from herbicides (0 in 1960 to 314 in 2007) (NRC 1996; Horrigan, Lawrence et al. 2002; Heap 2007; Yuan, Tranel et al. 2007). The current resolution to the increasing pesticide and herbicide resistant entities is the manufacture of new agrochemicals of which there is little information regarding their human health, wildlife and environmental impact (Carvalho 2006). Additionally, genetically modified organisms (GMOs) have been slowly added to the agricultural setting as a way of mitigating pesticide resistance in the last decade, although there are still many unanswered questions as to how this substitute will affect human and wildlife health and the overall environment (Nickson and Head 1999; Carvalho 2006). It is clear that like the overall chemical industry, the pesticide industry is an evolving technology that will continually pose challenging questions to scientists to counteract unwanted environmental and human health side effects.

Pesticide Exposure

Residues of pesticides can contaminate our foods either though direct consumption of fruits and vegetables or indirect consumption through fish and animals. They can linger in water and affect our drinking water and contaminate soils and even contribute to air contamination through "pesticide drift" from agricultural or residential spraying as depicted in Figure 1.3 below (Horrigan, Lawrence et al. 2002; Carvalho 2006). Furthermore, it is estimated that children consume between 45-108mg of soil per day while the 95% percentile is estimated to be as high as 1,751mg soil/day from various behavior patterns common in children such as *pica*, or deliberate soil ingestion (EPA 1997). Thus, children are especially likely to experience high levels of pesticide residue exposure.

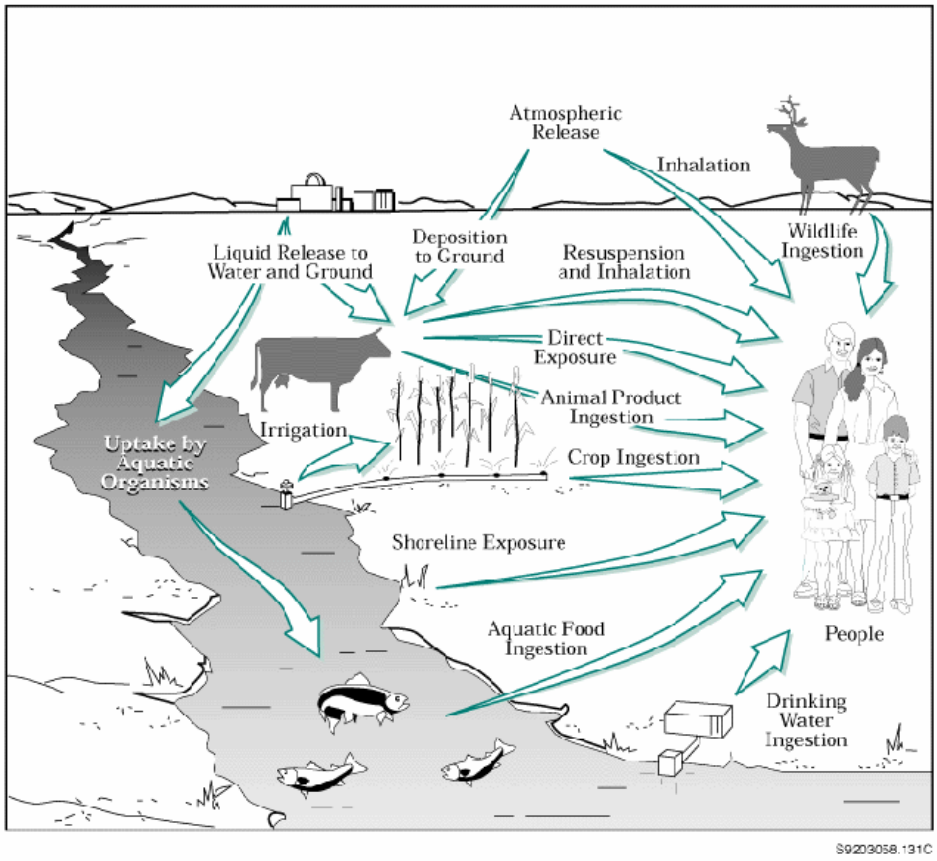


Figure 1.3 Exposure Pathways (DOE 1997).

From Figure 1.3 above, one can see that all environmental media and dietary sources could potentially contribute to pesticide exposure to humans and wildlife. Ideally, all sources of pesticide exposure would be evaluated to determine what exactly someone or something is exposed to at any given moment. However, this would be a tremendous and impractical task that is plainly not possible today. Accordingly, the focus of this dissertation will be limited in scope to one aspect of exposure analysis for the sake of time management. Therefore, this dissertation will only target the evaluation of soil and pesticide residues including parent pesticides and their degradation products.

Pesticides and their degradation products are typically found in soil either from direct application or indirect from crop spraying (NRC 1991). Adsorption is the main mode of interaction between pesticides in soils, although hydrogen bonding, ionic bonding and *Van der Waals forces* all play roles as well, albeit smaller and more pesticide specific roles (Howard 1991; Gevao, Semple et al. 2000; Ragnarsdottir 2000). The degree to which pesticides bind to soil is also determined by *ageing* of the chemical, or the time of contact between the chemical and soil and subsequent stronger association over time (Gevao, Semple et al. 2000). Additionally, the amount of organic (*humus)* versus inorganic content (minerals, rock fragments, etc.) present can affect how pesticides interact with soil (Ragnarsdottir 2000). All of the above interactions between soil and pesticides will ultimately determine how much of the pesticide residue is *bioavailable*, or the how much of the bound residue can be taken up by plants and/or wildlife and ultimately the human population (Gevao, Semple et al. 2000). These interactions are important to soil-pesticide relationships but are beyond the scope of this research;

however, explanations of the specific interactions of pesticides with different soil constituents are detailed elsewhere (Kramer 2001).

Environmental Monitoring for Pesticides

Environmental monitoring is performed to observe changes over time in baselines established for various environmental components (Wiersma 2004). Indicators of exposure are helpful in understanding the life cycles of chemicals as they are transported from its point of origin throughout the environment. More importantly, they can help determine routes and pathways of exposure for human health studies. More emphasis is placed on biological monitoring in modern day exposure assessments because biological monitoring has the advantage of determining body-burden concentrations that may help reflect target tissue concentrations accurately (Bradman and Whyatt 2005). Environmental monitoring represents a more tenuous relationship for human health assessments; the measurement of a chemical in the environment only defines the potential for exposure (Needham, Ozkaynak et al. 2005; Ryan 2007). However with contemporary, non-persistent pesticides that metabolize rapidly upon exposure, it is difficult to determine the degree of exposure with biological monitoring and therefore hard to establish causal relationships for any incidence except acute exposures, unless repeated measurements are performed (Bradman and Whyatt 2005; NRC 2006). The importance of environmental monitoring should not be diminished, however, but instead, be used to augment relationships suggested between pesticide exposure and adverse health outcomes from biomonitoring studies. A major criticism of CDCs annual National Report on Human Exposure to Environmental Chemicals is that the report does not include environmental monitoring data to support their biomonitoring data (NRC 2006). Recently, the National Children's Study has proposed the incorporation of mixed monitoring strategies (environmental, biological, questionnaires, etc.) over a large cohort

of children over a long time period in response to the President's Task Force on Environmental Health Risks and Safety to Children (Needham, Ozkaynak et al. 2005).

Analytical Chemistry

Biological and environmental monitoring requires high-quality methodologies to sustain confidence in the measurements they provide for exposure studies. In the post-World War II era of analytical techniques, demands were made on the scientific community to improve sample quality and measurements including better-quality sample integrity, higher throughput and superior quantification parameters (Raynie 2006). The complexity of environmental concerns has only added to these demands to push for fewer analytical methods with a greater number of toxicants in less time without sacrificing accuracy or precision. Analytical chemists are challenged with having to produce more modern and higher-technological methods while maintaining the analytical integrity of the sample and overall method.

Instrument technological advances have created some relief to the challenge of analytical method development. The idea of chromatography was first introduced in 1903 by Mikhail Tswett and his work with separation of plant pigments (Berezkin 2001). However, advancement of chromatographic techniques to enhance chemical analysis seriously began in the 1950s after World War II due to greater demands for clinical, environmental and synthetic organic chemical analysis (Laitinen 1989). The development of gas chromatography (GC) methods in 1952 had an immediate impact on the field of analytical chemistry; the petroleum industry, for example, saw the replacement of several, large distillation rooms replaced with GCs dedicated to separation of crude oil extracts (Laitinen 1989). Although the development of liquid chromatographic (LC) systems for separation of nonvolatile components began during

the 1950s and 1960s, they were overshadowed by the popularity of GCs and only became more prevalent during the 1970s (Laitinen 1989). As progression of smaller and faster computers advanced, so to did the field of mass spectrometry (MS) which made a great impact on the field of analytical chemistry as a versatile analysis detection tool. The first GC-MS units became available commercially during the late 1960s/early 1970s (Laitinen 1989). LC-MS systems proved more complex due to the need for solvent disposal prior to ionization and were not available commercially until the 1980s (Laitinen 1989). The development of atmospheric pressure ionization (API) in the 1980s greatly enhanced LC-MS capabilities (Whitehouse 1985; Hernandez, Sancho et al. 2005). Soon after this advancement in instrumentation, the number of LC-MS publications increased due in part to the universality of electrospray ionization (ESI) (Hernandez, Sancho et al. 2005). Additionally, higher sensitivity and selectivity requirements have led to more advanced mass analyzers in the past thirty years. Selective single ion monitoring (SIM) and selected reaction monitoring (SRM) with tandem mass spectrometry (MS-MS), have greatly enhanced these needs improving detection limits of analytical methods to lower than 1ng/mL (Hernandez, Sancho et al. 2005). Although MS-MS was introduced in 1968, its popularity really began in 1980 when the first triple-stage instruments were marketed by Finnigan and Sciex (Borman, Dagani et al. 1998). MS-MS techniques lead to highly selective and sensitive analyses that can contribute greatly to analytical measurements, specifically when requiring structural information (Willoughby 2002). Although constantly evolving sophisticated instrumentation will continue to give analytical method development for monitoring environmental toxicants certain

advantages, the basic method parameters that existed in the beginning are still as important today.

Method Development & Validation Performance Parameters

Conventional parameters common to analytical method development include terminology such as *sensitivity, selectivity, robustness, reproducibility,* etc. These terms help describe certain aspects of a method. The degree of uncertainty and consistency related to these terms signifies the quality of the method and therefore confidence in utilizing the method for field measurements (Taylor 1987). It is reasonable to believe that there will always be some uncertainty in chemical measurements, although it is the purpose of deliberate and prudent method development and validation to minimize uncertainty as much as possible. The typical flow of the development and validation of an analytical method is shown in Figure 1.4 below.

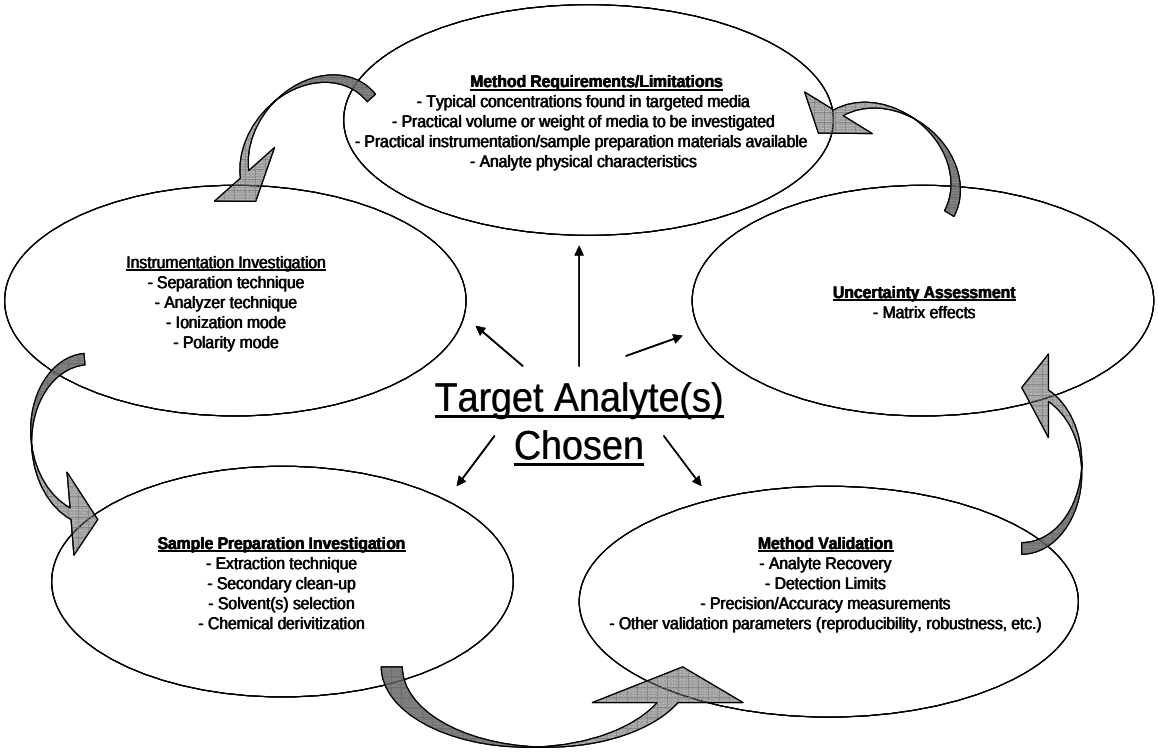


Figure 1.4 Analytical Method Development and Validation Flow Pathways

All method considerations are co-dependent. For example, the interesting target analytes, the chemicals selected and materials available will determine validation parameters. However, dependent on matrix effects and problems encountered, alternative methods may have to be re-investigated. Similarly, if a specific detection limit is desired for a certain application but the amount of sample available cannot yield that detection limit, re-evaluation of study parameters must be done. This multi-dimensional aspect of analytical method development can be daunting. However, a systematic approach to the complex process must be undertaken to achieve better-quality methods. The following table defines the terminology of specific parameters investigated in this research. Analyte recoveries, detection limits, quality control measurements and the linearity are all traditional parameters found in most method publications.

Validation Parameter Terminology

Traditional Parameters	
Analyte Recovery	This term defines the amount of starting material recovered after extraction divided by the amount of material spiked into a post-extraction sample and is expressed as a percentage.
Detection Limits (LOD)	Multiple methods for calculation. For this research, LODs will be defined as 3*SD at concentration 0. This is an experimentally derived value that is defined as the lowest measured value larger than the uncertainty of the method. Other sub-definitions are limit of quantification (LOQ) and instrument detection limit (IDL).
Quality Characterization (QC)	This term defines accuracy and precision of the analytical method. Relative standard deviations (RSDs) are measured for repeat measurements over time (repeatability/precision) and the degree of agreement between the measured values in samples compared to spiked concentrations in sample (accuracy).
Linearity	Ability to obtain test results that are directly proportional to the concentration of analyte in the sample.
Additional Parameters	
Specificity	Also referred to as efficiency. This defines the ability of the analytical method to assess unequivocally the analyte in the presence of components that may be expected to be present (*i.e*. matrix effects).
Robustness	The measure of the capcaity of a method to remain unaffected by small but deliberate variations in method parameters. Also referred to as reliability.

Table 1.1 Typical Method Validation Performance Parameters (Taylor 1987; Chan 2004; Baker, Olsson et al. 2005).

For the purposes of this research, traditional validation parameters will be assessed for the outlined analytical methods proposed below. Where appropriate, the additional parameters will also be investigated.

Proposed Method Development

Multiple pesticide residue analytical methods are proposed and developed in this research to investigate novel extraction and clean-up techniques, previously unanalyzed target analytes and/or improved method validation parameters in existing publications. The target analytes are a combination of contemporary and discontinued, although still present in people and the environment, insecticides and their degradation products in soil. Because degradation products are typically more polar and less volatile than their parent pesticide counterparts, the degradation products will be investigated using LC while the parent pesticides will be analyzed with GC. The outline for this research is shown below.

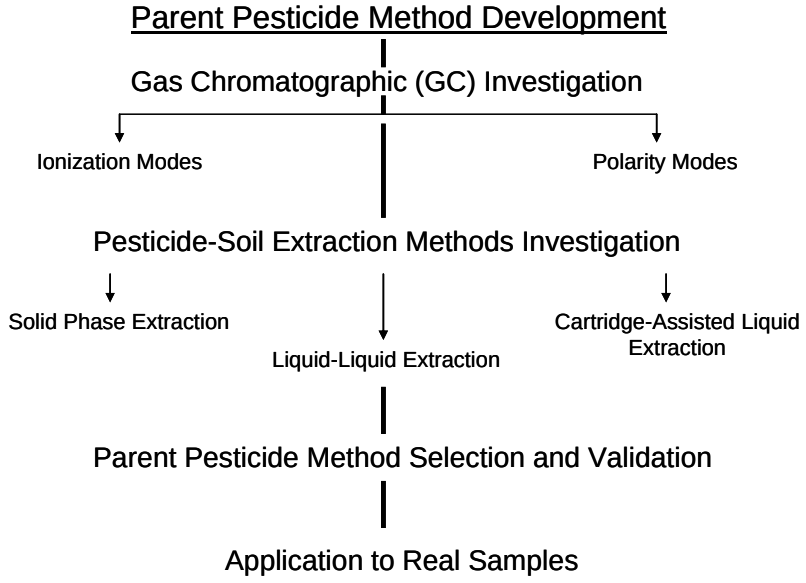


Figure 1.5 Proposed Parent Pesticide Method Development.

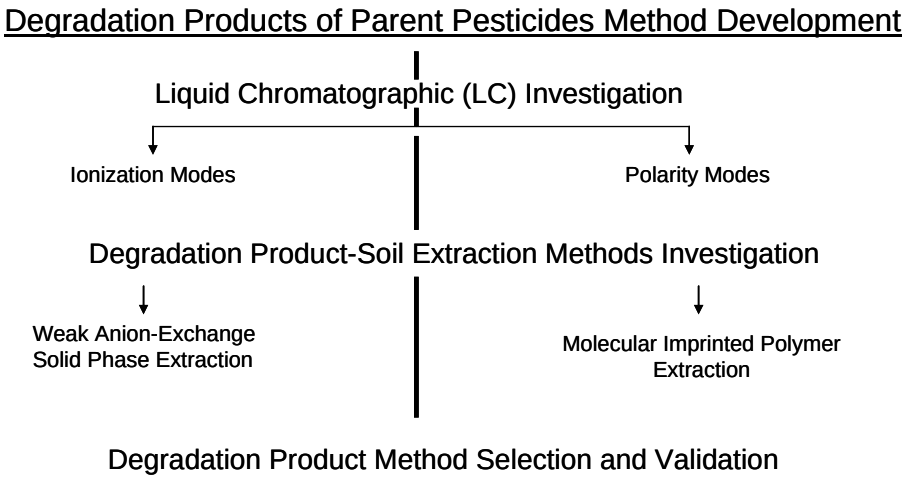


Figure 1.6 Proposed Pesticide Degradation Product Development.

CHAPTER 2: ANALYSIS OF PARENT PESTICIDES IN SOIL

Introduction

The ubiquitous characteristics of organochlorines pesticides prompted not only their prohibition in the 1970s but also many exposure studies to determine correlation between potential adverse health effects related to continuing exposures. For example, *p,p* DDT was found to be present in soils near the Yakima River in Washington State 25 years after application with no measured change in concentration, suggesting that these levels in conjunction with the high fish consumption of the surrounding populations could result in high DDT exposures throughout that timeframe (Johnson, Norton et al. 1988; Marien and Laflamme 1995). Another DDT chronic exposure study targeting a population on the Canary Islands showed higher median concentrations of DDT and metabolites in older inhabitants born prior to the DDT ban (total DDT burden 338ng/g body fat) than younger inhabitants born after the ban (110ng/g) (Zumbado, Goethals et al. 2005). The study also determined slightly higher concentrations of total DDT burden higher in women inhabitants (390ng/g) compared to men (340ng/g), a result they correlate with the high incidence of breast cancer found on the island (Zumbado, Goethals et al. 2005). Although DDT concentrations in the US have steadily decreased since its banning, DDE, a DDT metabolite, still remains in the adipose tissue of many Americans (Jaga and Dharmani 2003). The presence of persistent organochlorines pesticides still remains a topic of concern today and continual epidemiological, analytical and toxicological studies will be published in consequence of this enduring exposure.

The banning of persistent organochlorines motivated the pesticide industry to market less persistent pesticides. Organophosphates and pyrethroids are two classes of non-persistent

pesticides that have since emerged as frontrunners as the new generation of pesticides due to their lower toxicity to mammals and relatively shorter half lives in the environment. US EPA *Pesticide Usage and Sales Report* shows that organophosphates have steadily remained the foremost insecticide applied across all sectors from 1980-2001 (EPA 2001). The *National Home and Garden Pesticide Use Survey,* published in 1992 by Research Triangle Institution in conjunction with US EPA, states that pyrethroids are the most common and frequently applied home and garden insecticides (Whitmore 1992). With widespread use of non-persistent pesticides resulting in virtually all individuals exposed, there is increased interest regarding both acute and chronic toxicity of these pesticides, persistence in the environment, and their bioavailability.

In 2001, chlorpyrifos and diazinon were phased out for residential applications, due to observed fetal and newborn-stage adverse outcomes (impaired fetal growth and neurocognitive development) in rats and mice which was later reflected in human studies as well (low birth weight and birth length, small head circumference) (Spyker 1977; Muto, Lobelle et al. 1992; EPA 2000; EPA 2000; Berkowitz, Wetmur et al. 2004; Perera, Rauh et al. 2005; Whyatt, Camann et al. 2005). Epidemiological and toxicological studies correlating adverse health effects associated with pyrethroid chronic exposures are less prevalent because they are the most recent addition to the pesticide market and there is a general belief that they are less toxic to mammals. However, there have been recent findings of reproductive, endocrine and neurological adverse health effects associated with pyrethroid exposure that challenge this belief. A study in a pesticide factory in China found high correlations between ambient air fenvalerate concentrations

and decreased sperm count and mobility among directly exposed workers and indirectly exposed office workers versus workers at a separately located non-pesticide factory (Bian 2004). Bioallethrin showed 43% inhibition of testosterone from sex hormone binding globulin in human genital skin fibroblasts, an indication of endocrine disrupting abilities specifically relating to androgen activity (Eil and Nisula 1990). Studies showing increased prevalence of Parkinson's disease and pesticide exposure and/or usage have been supported by toxicological evidence of increased dopamine transporter-mediated dopamine uptake and increased levels of dopamine transporter protein itself (Stephenson 2000; Priyadarshi, Khuder et al. 2001; Gillette and Bloomquist 2003; Elwan, Richardson et al. 2006).

Another recent concern surrounding non-persistent pesticide applications is an increasing number of reported organophosphate and pyrethroid resistant pests, although this was also reported in the past with DDT but was a much rarer occurrence (NRC 1986). This has led to a growing trend of mixed organophosphate and pyrethroid applications. The advantage of dual pesticide applications is a synergistic effect when independent use is not effective, specifically in areas that have exhibited pyrethroid resistance (He, Chen et al. 2002; Martin, Ochou et al. 2003; Perry, Venners et al. 2007). As of 2001, at least 197 insecticides containing dual organophosphate and pyrethroid active ingredients are on the market in China (He, Chen et al. 2002). Bed-nets, usually treated with pyrethroids in malaria-endemic areas, have become increasingly ineffective against target pests with resistance (Miller, Lindsay et al. 1991; Shiff 2002; Darriet, Corbel et al. 2003). In the US, increasing resistance to permethrin used to treat head lice has led to multi-insecticide

applications (Yoon, Gao et al. 2003). However, it is now evident that even multiple-insecticide applications for head lice are cross-strain resistant (Downs, Stafford et al. 2000; Yoon, Gao et al. 2004). High levels of resistance to two pyrethroids, λ-cyhalothrin and deltamethrin, have been increasing as well with bed bug infestations across the US (Romero, Potter et al. 2007).

Due to growing evidence of chronic adverse health effects to non-persistent pesticides and increases in presence of total insecticides due to resistance and resulting multi-pesticide applications, there is a subsequent need for more accurate environmental and biological measures of exposure. Analytical methods to indicate acute and long-term exposures are a necessary part of understanding how pesticide use and exposure affect human health. Analyzing environmental media for the presence of pesticides is just one option for assessing health risk correlating to pesticide exposure. Although non-persistent pesticides break down more quickly in the environment as compared to the organochlorines pesticides, there presence is still apparent in environmental matrices weeks to months after application (Kamrin 1997; Roberts 1999). As such, these pesticides are almost certain to be found in soil as several studies have indicated. For example, Simcox *et.al*., determined levels of chlorpyrifos and other organophosphorus pesticides in both residential (non-occupational exposure) and agricultural (occupational exposure) homes (dust and soil) in eastern Washington State (Simcox, Fenske et al. 1995). Expectedly, chlorpyrifos levels were higher in homes where occupationally exposed workers live although there were detects for organophosphorus pesticides in homes where the non-occupationally exposed workers lived as well. Interestingly, their

results indicated a high correlation between OP residues found in soil to even higher levels found in dust samples inside the homes. This result indicates that soil sampling has dual importance to understanding exposures. Not only can soil be an indicator of what adults and children are exposed to outside the home but also what potential exposures they may encounter inside the home because of possible residue transfer from outside the home to the inside. Thus, soil is an indicative matrix to assess pesticide exposure. Soil is also an inexpensive and accessible matrix and consequently used for many pesticide exposure analyses. Soil is especially important for understanding children's exposure to pesticides since children typically spend more time outdoors than adults (Landrigan 2004). Their time spent outdoors is usually accompanied by more time spent either directly on the ground or in proximity according to their shorter heights and tendency to pica (EPA 1998; Wilson, Chuang et al. 2003). The potential health risk associated to soil exposure pathways for children has been estimated to be 12 times that of adults (Simcox, Fenske et al. 1995).

Analytical Chemistry

There are various analytical techniques used to extract pesticides from soil to determine exposure. Soxhlet, sonication and shake-flask soil extraction are the most commonly used methods to extract pesticides due to feasibility and low cost. Recently, soil extraction methods for organic pollutants have focused on sonication, microwave extraction, supercritical fluid and pressurized liquid extractions as detailed in Table 2.1. Generally, these methods use less solvent, have quicker extraction times but are typically more expensive.

YEAR	ANALYTES OF INTEREST	SAMPLE SIZE	EXTRACTION METHOD	SECOND CLEAN-UP	ANALYSIS	LOD (ng/g)	RECOVERY (%)	RSD (%)	AUTHOR
2001	OPs, OCs, Pyrethroids	5g	Sonication	-	GC-MSD	-	99-108	0.9-5.4	Castro et al., Journal of Chromatography A (2001)
2002	OPs, OCs	5g	PLE	SPE (Alumina, Na_2SO_4, Cu)	GC-MSD	3.9-4.0	90-130	2.0-12.0)	Dabrowski et al., Journal of Chromatography A (2002)
2003	OPs, OCs	5g	PLE	3 SPE (Ph+C-18+Alumina)	GC-MSD	-	66-116	4.0-16.0	Dabrowska et al., Journal of Chromatography A (2003)
2003	OPs, OCs	2-4g	Sonciation	-	GC-ECD	3.6-15.1	69-109	2.0-7.0	Lyytikainen et al., Archives of Environ. Contam. Toxicology (2003)
2003	Pyrethroids	10g	Sonication	-	GC-MSD	-	22-149	-	Wang et al., Pedosphere (2003)
2003	OPs, OCs, Pyrethroids	5g	Shake-Flask	-	GC-AED	2.3-4.0	100-104	2.2-8.2	Vinas et al., Journal of Agric. Food Chem. (2003)
2004	OCs	1g	PLE	Carbon SPE	GC-ECD	-	92-141	4.0-28.0	Concha-Grana et al., Journal of Chromatography A (2004)
2004	OPs, OCs, Pyrethroids	20g	Sonication	anhydrous $MgSO_4$/deactivated Florisil	GC-ECD	0.22-0.67	94-104	3.6-10.5	You et al., Archives of Environ. Contam. Toxicology (2004)
2004	OPs, OCs, Pyrethroids	5g	Sonication	-	GC-MSD	0.2-1.5	87-98	3.7-9.5	Sanchez-Brunete et al., Journal of Agric. Food Chem. (2004)
2004	Pyrethroids	2g	Microwave	Florisil column	GC-ECD/ GC-MSD	1.0-3.0 (ECD) 0.3-0.7 (MSD)	101-107	1.0-6.0	Esteve-Turrillas et al., Analytica Chimica Acta (2004)
2005	OPs, OCs, Pyrethroids	5g	Sonication	anhydrous Na_2SO_4	GC-MSD	0.05-4	74-111	3.6-13.7	Goncalves et al., Talanta (2005)
2005	OPs, Pyrethroids	5g	Sonication	centrifiguation with DCM	GC-NPD/ GC-MSD	0.2-9.8	98-104	2.2-6.2	Fenoll et al., Journal of Agric. Food Chem. (2005)
2005	OPs, OCs, Pyrethroids	25g	Shake-Flask	-	GC-ECD/ GC-MSD	-	51-69	3.8-5.9	Rissato et al., Journal of Agric. Food Chem. (2005)
2005	OPs, OCs, Pyrethroids	6g	SFE	C-18 SPE	GC-ECD/ GC-MSD	-	77-268	4.7-9.1	Rissato et al., Journal of Agric. Food Chem. (2005)
2006	Pyrethroids	2g	Microwave	Florisil column	GC-EI/MS/MS	0.15-0.47	95-120	3.0-20.0	Esteve-Turrillas et al., Anal. Bioanal. Chem. (2006)

Table 2.1 Summary of Soil/Sediment Extraction Methods since 2000.

As technology improves, innovative alternative methods are being introduced to the market that require shorter extraction times or have high selectivity. For example, solid-phase microextraction (SPME) has been investigated for soil extraction over the past decade because of its low solvent usage, low cost and simplicity (Beltran, Lopez et al. 2000). Headspace SPME has been utilized to investigate semi-volatile or volatile

pesticides from solid matrices but is ineffective for non-volatile, polar compounds. Direct-immersion SPME has also been investigated with solid samples but is either limited to a select few analytes of interest or encounters problems with fiber stability and carryover (Beltran, Lopez et al. 2000). Additionally, agrochemical immunoanalysis is a new progression in alternative extraction techniques. Although these immuno-based extractions are more popular with biomonitoring studies, their use in environmental monitoring has increased in the past decade. Development of a tracer, or antibody, that is chemically modified for methyl parathion has been developed for quantitative detection in soil with a low detection limit of 15 parts-per-billion (ppb) and high recoveries 85-110% (Kolosova, Park et al. 2003). An enzyme-linked immunosorbent assay (ELISA) has been reported for its extraction with bioallethrin in food, soil and dust samples with 100% recovery in soil (Kaware, Bronshtein et al. 2006). Detection limits were not reported, however recovery samples were shown as reproducible at 10ng/mL (ppb). Despite the high recoveries and low detection limits associated with these specific-tracer extraction methods, they are highly selective for on only one pesticide and would be impractical for multi-analyte extractions. In this work, the use of a novel secondary clean-up for environmental analysis is used. ChemElut® cartridges consist of immobilized hydromatrix material to assist in liquid-liquid extractions which allows for possible automation. This extraction cartridge uses the hydromatrix-immobilized sorbent to avoid emulsion formation that is sometimes a problem with traditional liquid-liquid extractions. In addition, extraction is performed with solvent flow by gravity and not applied force, which can improve precision of the extraction recoveries (Varian 1993). Cartridge assisted liquid-liquid extraction has largely been employed for biological

samples (example: urinary analysis for the presence of organophosphorus metabolites) and has not been applied to environmental pesticide residue analysis.

Complete analysis and interpretation of all validation parameters detailed in the introduction were performed on a collective group of non-persistent contemporary pesticides in soil. An accelerated solvent extraction (ASE) method was developed followed by a secondary clean-up for quantifying six organophosphates, seven pyrethroids and seven organochlorines shown in Figure 2.1 in soil.

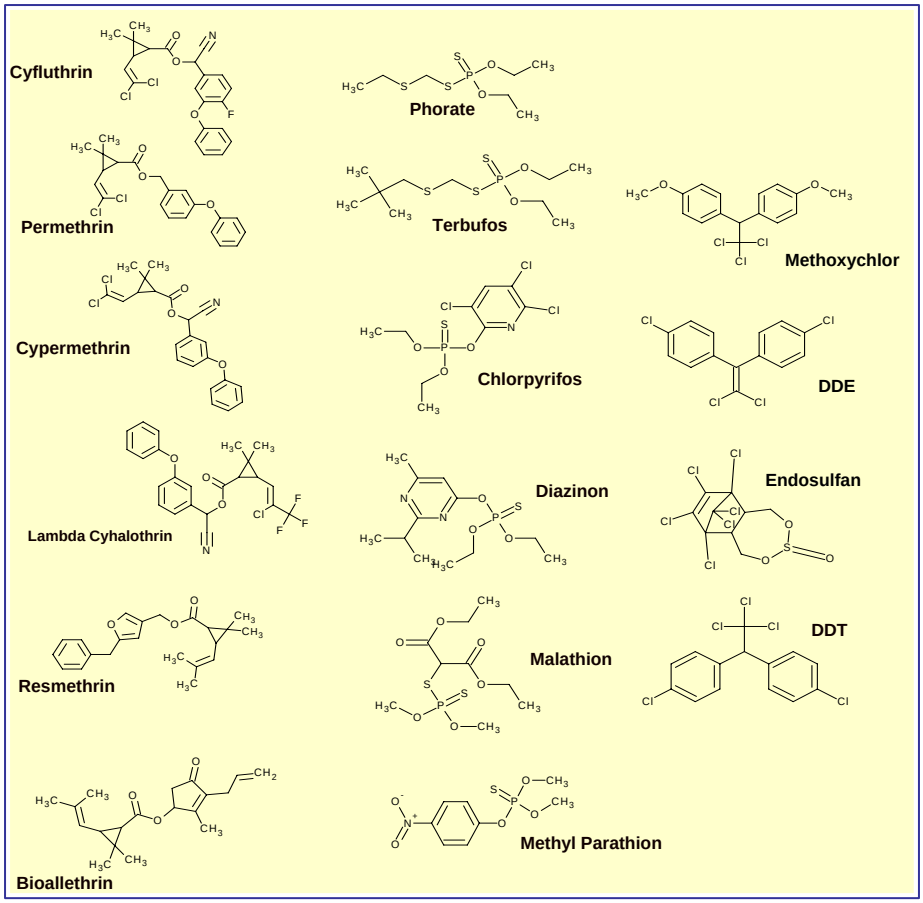


Figure 2.1 Target Parent Pesticides for Method Development.

Several aspects of the extraction and clean-up system were investigated and optimized to achieve the most precise, accurate and robust method for the target analytes. Table 2.2 details important physical and chemical properties of these analytes that represent important determinants for analytical methodology; all values were determined using ChemAxon© MarvinSketch version 1.4.6 (Appendix 2A).

					pH3 (25ºC)		pH4 (25ºC)		pH5 (25ºC)		pH6 (25ºC)		pH7 (25ºC)	
	ANALYTE	MOLECULAR WEIGHT	pKa[a]	Water Solubility[c] (ppm)	Charge State[b]	log P	Charge State[b]	log P	Charge State[b]	log P	Charge State[b]	log P	Charge State[b]	log P
PYRETHROIDS	Cyfluthrin	434.29	10.31	(~.000001)	Neutral	5.44	Neutral	5.44	Neutral	5.44	Neutral	5.44	Neutral	5.44
	Permethrin	391.29	n/a	(<1)	Neutral	5.73	Neutral	5.73	Neutral	5.73	Neutral	5.73	Neutral	5.73
	Cypermethrin	416.30	10.62	Insoluble	Neutral	5.30	Neutral	5.30	Neutral	5.30	Neutral	5.30	Neutral	5.30
	Lambda Cyhalothrin	449.86	10.62	(~0.005)	Neutral	5.15	Neutral	5.15	Neutral	5.15	Neutral	5.15	Neutral	5.15
	Resmethrin	338.45	n/a	Insoluble	Neutral	4.99	Neutral	4.99	Neutral	4.99	Neutral	4.99	Neutral	4.99
	Bioallethrin	302.41	n/a	Insoluble	Neutral	4.05	Neutral	4.05	Neutral	4.05	Neutral	4.05	Neutral	4.05
ORGANOPHOSPHATES	Phorate	260.36	n/a	(~50)	Neutral	3.63	Neutral	3.63	Neutral	3.63	Neutral	3.63	Neutral	3.63
	Terbufos	288.42	n/a	(10-15)	Neutral	5.00	Neutral	5.00	Neutral	5.00	Neutral	5.00	Neutral	5.00
	Chlorpyrifos	350.58	n/a	(~2)	Neutral	4.97	Neutral	4.97	Neutral	4.97	Neutral	4.97	Neutral	4.97
	Diazinon	304.34	3.19	(~40)	Partially Ionized	4.10	Partially Ionized	4.40	Neutral	4.50	Neutral	4.50	Neutral	4.51
	Malathion	330.35	13.41	(~145)	Neutral	1.91	Neutral	1.91	Neutral	1.91	Neutral	1.91	Neutral	1.91
	Methyl Parathion	263.20	n/a	(~50)	Neutral	0.76	Neutral	0.76	Neutral	0.76	Neutral	0.76	Neutral	0.76
ORGANOCHLORINES	Methoxychlor	345.65	n/a	Insoluble	Neutral	4.85	Neutral	4.85	Neutral	4.85	Neutral	4.85	Neutral	4.85
	DDE	318.03	n/a	Insoluble	Neutral	5.83	Neutral	5.83	Neutral	5.83	Neutral	5.83	Neutral	5.83
	DDT	354.49	n/a	Insoluble	Neutral	6.39	Neutral	6.39	Neutral	6.39	Neutral	6.39	Neutral	6.39
	Endosulfan	406.92	n/a	Insoluble	Neutral	1.95	Neutral	1.95	Neutral	1.95	Neutral	1.95	Neutral	1.95

[a] Calculated from ChemAxon© (Other minor pKas may exist but are negligible to this research)
[b] Estimated based on pKas
Log D estimates
[c] Reported at 20ºC or 25ºC

Table 2.2 Physical and Chemical Properties of Target Pesticides (ExToxNet 1997; ChemAxon 1998; O'Neil 2001).

Because the target analytes are neutral at pH environments studied in this research (excluding diazinon that shows partial ionization at pH3, 4), their partition behavior will show tendency to favor organic phases. This collective hydrophobic property will serve as the basis to begin extraction analysis. First, development of the ASE system will be discussed, specifically the various solvent systems used to extract soil. Secondly, multiple parameters for the additional clean-up steps were evaluated including solvent selection, cartridge selection, cartridge rinse selection and volume of solvent used. Lastly, method validation will be detailed including complete analyte recovery, detection limits and quality control characterization. The results of these investigations indicate that it is possible to develop a single robust method capable of analyzing multi-class

pesticides using a small sample with excellent recovery, sensitivity, and precision thus exemplifying the utility of this method for analyzing environmental media to indicate possible pesticide exposures.

Method Development

Standards and Curve Concentrations

Unfortunately, not all target analytes had corresponding label internal standards. However, one appropriate label internal standard was used per pesticide class that is physically and chemically similar to target analytes in that class as shown in Table 2.3. This was done to reduce quantitation error associated with using dissimilar label and/or surrogate standards as internal standards for quantitation.

Individual standard stock solutions were prepared by weighing between 2-5 mg of each native analyte into a vial with 15 mL of acetonitrile. The working standard solution 1 was prepared by diluting each starting stock solution to approximately 9µg/mL in 16 mL acetonitrile. The working standard solution 2 was prepared by diluting working standard solution 1 to 1 µg/mL in acetonitrile. Calibration standards were prepared by spiking aliquots of working standard solution 2 and 1 into 15 mL acetonitrile (Table 2.4).

Analyte	Pesticide Class	Internal Standard
Phorate Terbufos Diazinon Methyl parathion Malathion Chlorpyrifos	Organophosphate	Chlorpyrifos methyl-d6
op DDE *pp* DDE *op* DDT *pp* DDT Endosulfan *alpha* Endosulfan *beta* Methoxychlor	Organochlorine	Endosulfan *alpha* -d_4
Bioallethrin Resmethrin Lambda cyhalothrin *cis* Permethrin *trans* Permethrin Cyfluthrin Cypermethrin	Pyrethroid	Permethrin-phenoxy-$^{13}C_6$ (cis/trans mix)

Table 2.3 Native Pesticide Analytes and Corresponding Label Standard.

Standard	Volume of Dilution	Dilution	Concentration
	(µL)	(Working solution)	(ng/mL)
1	75	2	5
2	150	2	10
3	300	2	20
4	750	2	50
5	167	1	100
6	334	1	200

Table 2.4 Calibration Curve Standard Preparation.

Starting stock solutions of each isotopically labeled standard were prepared by weighing approximately 2.5 mg of each labeled standard with 15 mL acetonitrile. Working internal standard solution 1 was prepared by diluting each starting stock solution with 15 mL acetonitrile to generate a resulting concentration of 50 µg/mL. Working internal standard solution 2 was prepared by diluting working standard solution 1 to 1 µg/mL in 15 mL acetonitrile. All stock and working solutions were stored at -20°C until needed.

Instrumental Analysis

Gas chromatography analysis would be successful for the target analytes chosen as they are semi-volatile in nature. Consequently, only GC analysis was chosen for separation and analysis for the following method development and validation procedures. Mass spectrometry was chosen as the detection tool for quantitation because of its versatility and selectivity for multi-component analysis. Electron ionization (EI) and chemical ionization (CI) are two common ionization sources that are used to interface chromatography effluent and mass spectrometry analysis. EI is considered a harsher ionization because it induces extensive fragmentation and sometimes complete fragmentation of the molecular ion, a situation that may be undesirable for analyses that

require molecular ion detection and quantitation (De Hoffman 2002). CI offers an alternative softer ionization technique which yields less fragmentation of the molecular ion species (De Hoffman 2002). All target analytes and label internal standards were investigated with both EI and CI, using methane gas, to observe ionization patterns needed for quantitation. Furthermore, both negative and positive polarity modes were assessed with both EI and CI techniques.

Using solvent standards at concentrations of 1ng/µL (ppm), 1µL of each standard (in toluene) was injected into the GC-MS (TSQ, ThermoFinnigan, San Jose, CA) to observe retention time and separation behavior. Additionally, all samples were monitored using a SCAN analysis to observe molecular ion and fragmentation ion species generated as a result of both EI and CI techniques. All samples were injected by autosampler (CTC A200S, Carrboro, NC). The capillary column used for separation of analytes was an ultra-low bleed 30-m Factor Four (Varian) VF-5MS (5% phenyl, 95% dimethylpolysiloxane) with 0.25µm film thickness and 0.25 mm ID. Initially, a simple GC program was used starting at 80ºC, held for 2 minutes and then ramped at 10ºC/minute to 300ºC and held for 6 minutes for a total runtime of 30 minutes (injector-300ºC and transfer line 310ºC).

Electron ionization in positive mode was chosen as the ionization technique for all of the target analytes as it resulted in ionization patterns adequate for successful quantitation. Chemical ionization failed to produce fragmentary ions for several of the target analytes thus limiting monitoring of additional ions, or confirmation ions, an advantage for

quantitative analysis. Additionally, negative mode ionization failed to generate suitable spectra for analysis for many target analytes as shown in Figure 2.2 where methoxychlor is not detected.

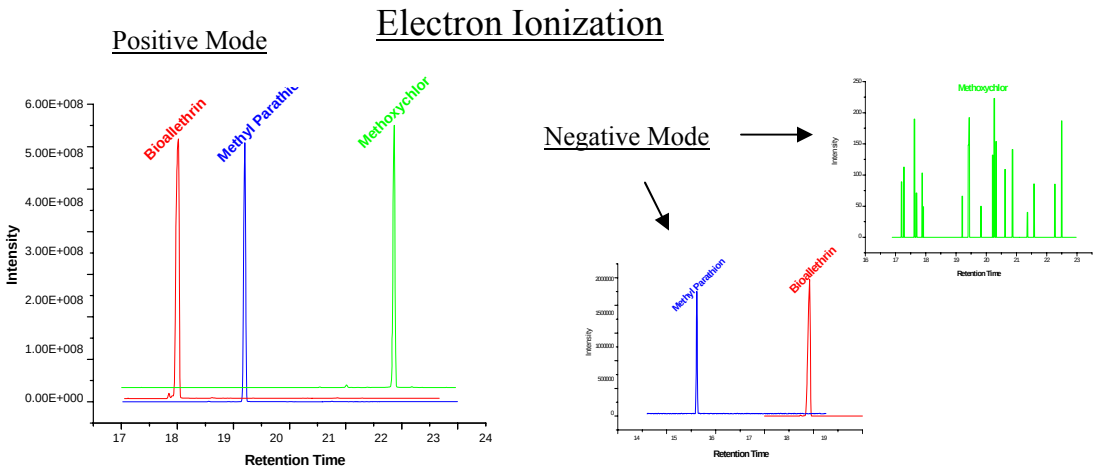


Figure 2.2 Electron Ionization Spectra for Selected Analytes.

After abundant molecular and/or fragment ions were chosen for monitoring, target analytes were analyzed in single ion monitoring mode as detailed in Table 2.5. All ions were produced by electron ionization (70eV) and resulted in 9 SIM windows. Analysis was performed with at least two ions monitored for each analyte, except for the permethrin isomers where the only fragment ion produced with adequate intensity was *m/z* 183. The acquisition software used to quantify target analytes was Xcalibur® version 1.3 (ThermoFinnigan, San Jose, CA).

Pesticide Class	Pesticide	Monitored Ions (*m/z*)	SIM Group
Organophosphate	Phorate	231,260	1
	Terbufos	231,288	1
	Diazinon	137,304	1
	Methyl parathion	109,263	2
	Chlorpyrifos methyl (L)	292, 296	2
	Malathion	158,173	3
	Chlorpyrifos	258,286	3
Organochlorine	*op* DDE	246, 318	5
	pp DDE	246, 318	5
	op DDT	165,235	6
	pp DDT	165,235	6
	Endosulfan *alpha*	195,239	5
	Endosulfan *alpha* (L)	237,343	5
	Endosulfan *beta*	195,237,339	6
	Methoxychlor	227,344	7
Pyrethroid	Bioallethrin	123,136	4
	Resmethrin	123,171	6
	Lambda cyhalothrin	181,449	7
	cis Permethrin	183	8
	trans Permethrin	183	8
	cis/trans Permethrin (L)	163,189	8
	Cyfluthrin	163,206	9
	Cypermethrin	163,181	9

L, Labeled compounds

Table 2.5 Single Ion Monitoring Specifications of Target Analytes.

Co-elution of certain analytes was problematic with many of the pyrethroids with multiple isomers, a typical problem with pyrethroid isomers unless an enantioselective column is used for separation (Liu and Gan 2004). Lambda cyhalothrin, cyfluthrin, bioallethrin and cypermethrin were all integrated and quantitated as aggregate isomers since they were unable to be separated chromatographically. In addition, *alpha*-endosulfan and label *alpha*-endosulfan co-eluted, a problem easily corrected by slowing down the oven temperature program. As a result, the new GC program began at 80°C for two minutes and ramped at 10°/minute to 210°C, ramped at 1°C/minute to 217°C and lastly to 300°C at 10°C/minute and held for one minute yielding a total runtime of 31.3 minutes. Only semi-separation of the two analytes was achieved using this newer GC program (only with a runtime >70 minutes did the two analytes finally separate).

However, the two analytes are monitored at different *m/z* ratios so should not affect quantitation.

Figure 2.3 shown below illustrates a total ion chromatogram using the final GC program. Separation by SIM group with appropriate mass filters is shown below in Figure 2.4.

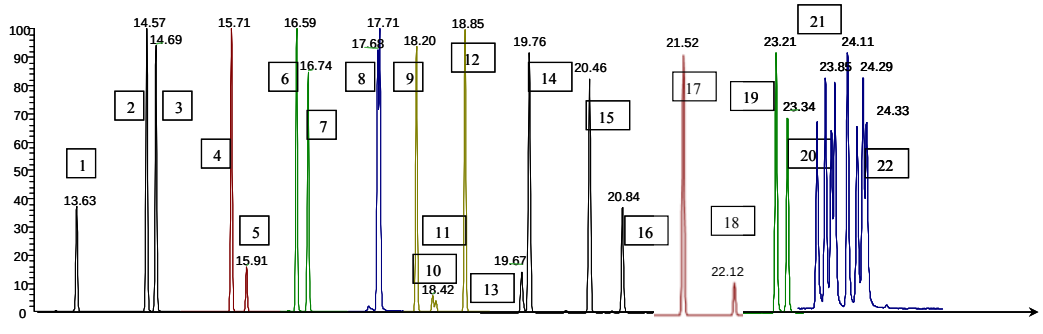


Figure 2.3 Total Ion Chromatogram of Target Analytes. Peaks are as follows: (1) phorate, (2) terbufos, (3) diazinon, (4) label methyl chlorpyrifos, (5) methyl parathion, (6) label methyl chlorpyrifos, (7) chlorpyrifos, (8) bioallethrin isomers, (9) *op*-DDE, (10) label *alpha*-endosulfan, (11) *alpha*-endosulfan, (12) *pp*-DDE, (13) *beta*-endosulfan, (14) *op*-DDT, (15) *pp*-DDT, (16) resmethrin, (17) methoxychlor, (18) lambda cyhalothrin isomers, (19) *cis*-permethrin (label + native), (20) *trans*-permethrin (label + native), (21) cyfluthrin isomers, (22) cypermethrin isomers

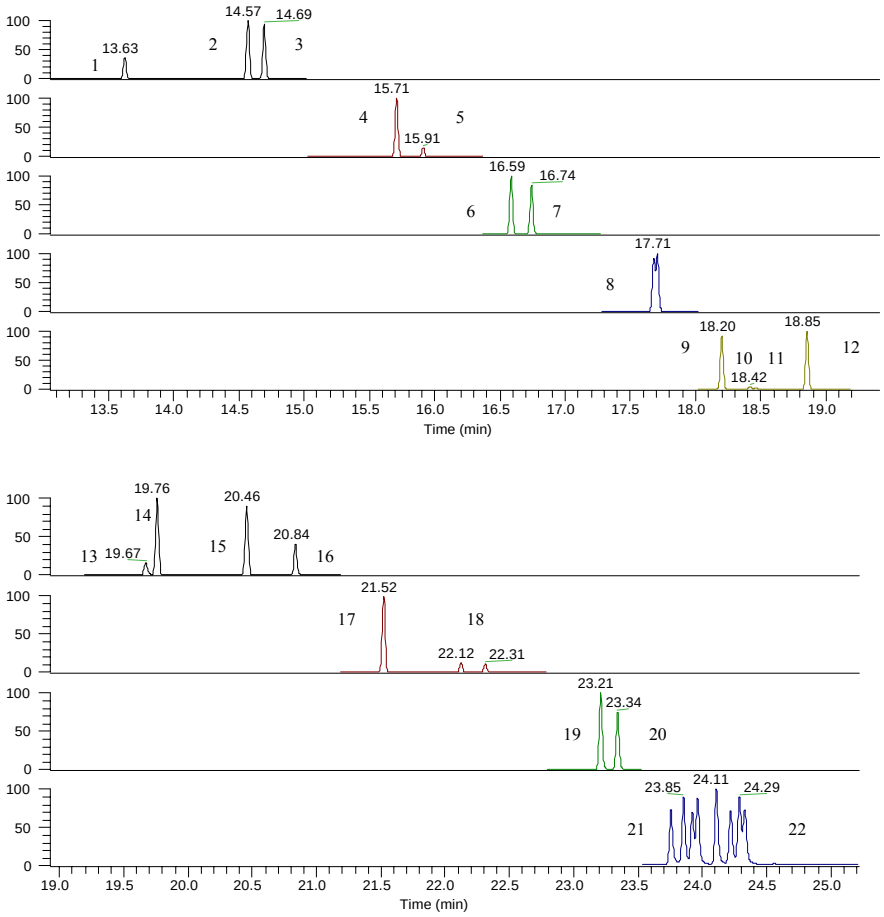


Figure 2.4 SIM for Target Analytes Separated by Mass Filters.

Soil and Soil Fortification

All soil samples used for development and validation of subsequent experimentation were drawn from the Soil Library at the Emory University Environmental Chemistry Laboratory. Specific samples used were from the Baltimore, Maryland area and are representative of typical backyard composite soil samples from this area. Approximately 1 g of soil was spiked with various concentration of native standard into a 10 mL glass vial. 3 mL of hexane was then added to the sample. The sample was vortexed, capped and shaken for 12 hours using a high-power shaker (Gilson-Middleton, WI). The

samples were then evaporated using the TurboVap (Zymark, Hopkinton, MA) at 30°C and 5psi for 10 minutes.

Accelerated Solvent Extraction

Accelerated solvent extraction was chosen as the extraction method for the target analytes due to rapid analysis times, low labor intensity and high recoveries with pesticides from soil and/or sediment as seen with previous studies (Dabrowski, Giergielewicz-Mozajska et al. 2002; Dabrowska, Dabrowski et al. 2003; Concha-Grana, Turnes-Carous et al. 2004). ASE was performed using an ASE 200 System (Dionex, Sunnyvale, CA) with 11 mL extraction cells. After a cellulose filter was packed into an 11 mL extraction cell, approximately 1 g of soil was added on top of the filter in the cell. Each sample was then spiked with 100 ng/g of internal standard. Florisil® (Sigma Aldrich) was filled on top of the soil sample about 3 mm from the top of the extraction cell. An additional cellulose filter was packed on top of the Florisil® and the cell was capped and inverted onto the ASE 200 carousel.

The selection of Florisil® as a filler agent for the extraction cells was determined by analyzing three different agents to use as an additional clean-up step for reduction of contaminating peaks in the analysis. Silica gel (JT Baker), Ottawa sand standard (Fisher Scientific) and Florisil® were all analyzed to see which filler agent produced the cleanest chromatograms. While sand reduced the least amount of contamination overall, Florisil® suppressed most contamination without interfering with analytes of interest and silica gel

actually increased contamination as shown using methyl parathion and lambda cyhalothrin as examples in Figure 2.5.

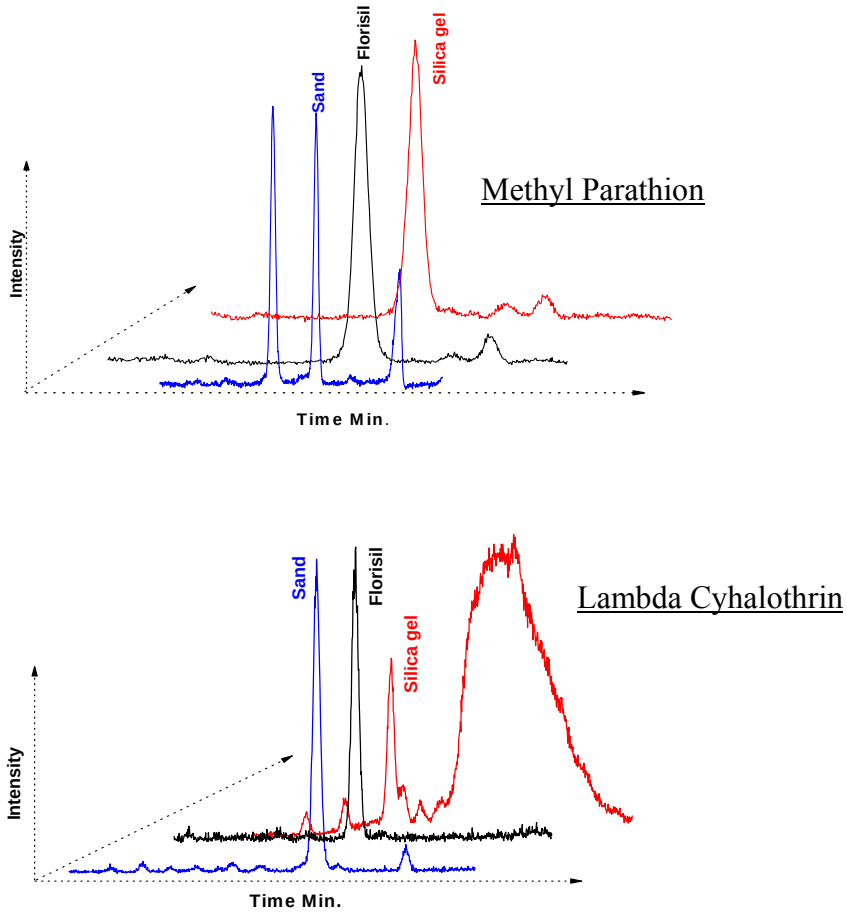


Figure 2.5 Chromatographic Results for Methyl Parathion and Lambda Cyhalothrin with Different ASE Fillers.

Prior to analyzing soil extracts, blank samples were prepared and analyzed to determine any contamination from the ASE cell itself. These samples were prepared with cellulose filters and Florisil® which completely filled the extraction cell. The extracts were evaporated, reconstituted and injected using full SCAN mode into the GC-MS. Chromatograms resulted in many interfering peaks present in the blanks that consisted of mainly silicones and fatty acids (as determined with Xcalibur® Library Browser). To

reduce this contamination, all extraction cells were sonicated in a series of solvents (water, methanol, acetone, hexane) for 45 minutes each prior to each sample set which appeared to reduce many of the peaks seen prior to cleaning.

Selection of the ASE extraction method (pressure and temperature settings) was taken from manufacturer recommendations for measuring organochlorines in sediment and fish tissues (EPA 1980). The extraction cycle for each soil sample was 5 minutes which consisted of heating the extraction cell to 100°C and pressurizing the solvent system to 1500 psi. Two static cycles were employed with the first cycle flushing 50% of the solvent in the cell into the collection cell and replenishing the cell with additional solvent. Total extraction time was 15 minutes per sample with approximately 15 mL of extracted solvent collected in each collection vial.

The solvent chosen for ASE extraction was taken from several studies that collectively decided that the presence of water with soil extractions helps improve recoveries of ionic and non-ionic pesticides by breaking down hydrogen bonds and therefore the structure of the soil allowing the organic solvent a greater surface area to extract (Dean 1999). Two different solvent systems were investigated using ASE: aqueous acetonitrile and acidic aqueous acetonitrile. A summary of results showing the average recovery ($n=5$) is shown in Figure 2.6 Target analytes recovered from soil were normalized to "100%" samples spiked after extraction just prior to analysis.

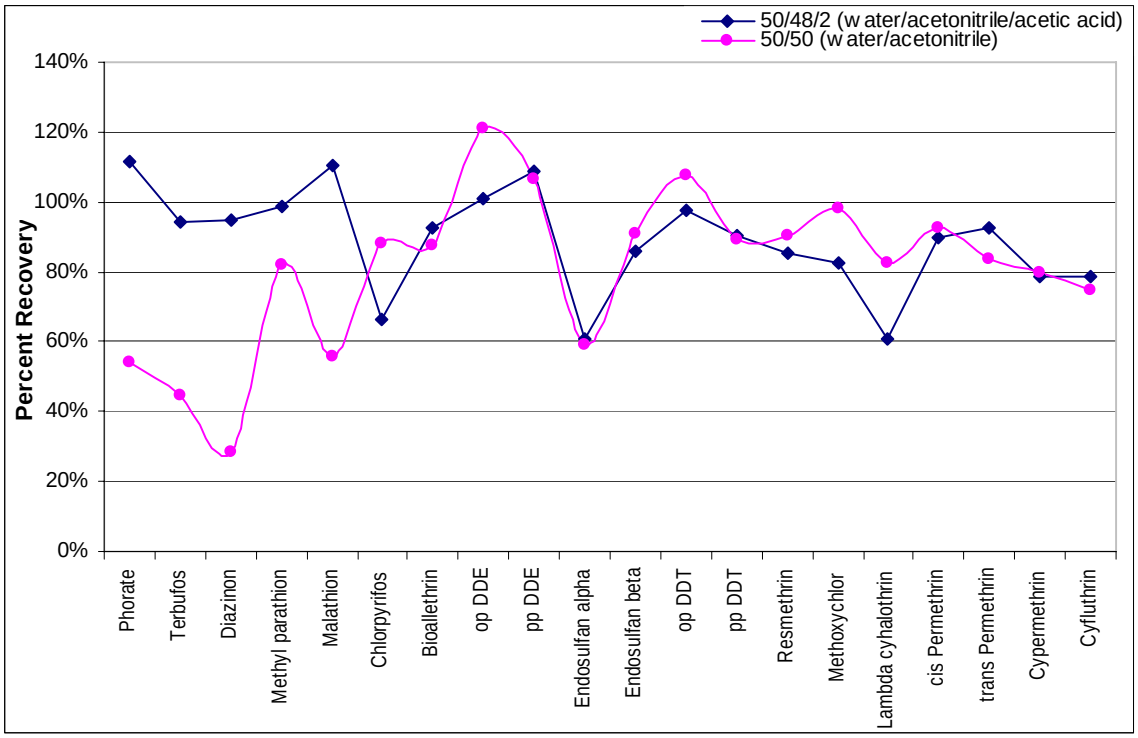


Figure 2.6 Percent Recovery for Target Analytes Comparing 2 ASE Extraction Solvent Systems.

As expected, the addition of acid to the extraction solvent resulted in no change for recoveries of the organochlorines and pyrethroids. However, the acidic aqueous acetonitrile extraction solvent as the extraction solvent yielded higher recoveries for all of the organophosphates, except chlorpyrifos. This was unexpected because all of the organophosphates are neutral at the pH of the extraction solvent (pH~3) with the exception of diazinon. Diazinon, a basic compound with a pKa = 3.19, is partially ionized at the extraction solvent pH. The part neutral, part ionic state of diazinon could be beneficial in environments where both aqueous and organic phases are present, thus increasing recovery. Without the addition of acetic acid, the pH of the extraction solvent is approximately 6 and thus diazinon would remain neutral and may be expected to respond similarly to the other organophosphates. The mechanism for increased recovery

of organophosphates with the lower pH adjustment is unknown, however, one possible hypothesis is considered. It has been demonstrated that with increased pH, hydrolysis rates of organophosphates is increased due to OH groups causing nucleophilic attack on the electrophilic phosphorus atom (Coulibaly and Smith 1994; Ragnarsdottir 2000). Methyl parathion was studied under laboratory conditions in pH environments 3-11 (at constant temperature) and half-life measured in days. At pH3, the half-life of methyl parathion is ~175 days compared to pH7 at 108 days (half-life at pH 6 was not measured). Conceivably, this would not have any affect on extraction recoveries as laboratory extractions are performed in hours and thus hydrolysis rates are too slow to affect extraction recoveries. But, it has also been demonstrated that increased pH and increased temperature have a combined effect with much more rapid hydrolysis rates (in minutes) than individual parameter investigated alone (Coulibaly and Smith 1994; Ragnarsdottir 2000). Consequently, the high temperature used for ASE extraction (100°C) in conjunction with a higher pH could actually enhance decomposition of the organophosphates more rapidly than in low pH environments. If this were true, more of the intact organophosphate would be present at lower pH and therefore available for extraction. Interestingly, this effect would be less observable if label internal standards were used for each organophosphate because the label would mimic the native target analyte and ratios would be similar in both pH environments. The label internal standard used for the organophosphates is chlorpyrifos methyl, which is most similar in physical structure and chemical behavior to that of the target analyte, chlorpyrifos. The increased recoveries seen with the other OPs at lower pH are not reflected with chlorpyrifos, reasonably due to the inclusion of the label chlorpyrifos for quantitation.

To observe recovery in correlation to the aqueous/organic ratio of the extraction solvent, three solvent systems were evaluated: 30%, 50%, and 70% water in acidic acetonitrile. Results are summarized in Figure 2.7 showing averaged recoveries (n=5).

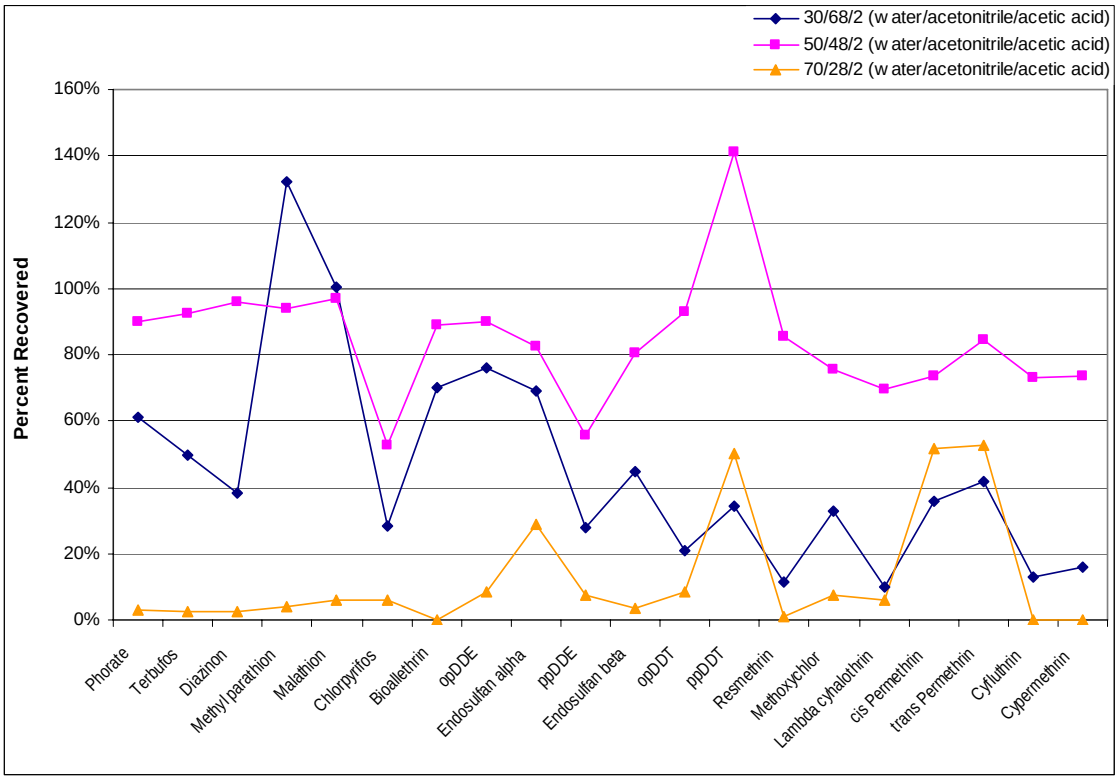


Figure 2.7 Percent Recovery for Target Analytes Comparing Various Organic/Aqueous Portions of ASE Extraction Solvent.

Collectively, the analytes showed higher recoveries with increased portions of acetonitrile in the extraction solvent which was expected with neutral analytes at pH~3. For subsequent experimentation, the extraction solvent consisting of 50/48/2 water, acetonitrile and acetic acid respectively was chosen. However, problems were encountered when attempting to pre-concentrate the extraction solvent for analysis (evaporation time > 8 hours) due to lowered vapor pressure with the presence of water

and acetic acid. Therefore, in an attempt to decrease evaporation time, extraction with 100% acetonitrile was performed and compared to the aqueous acidic acetonitrile extraction solvent recoveries. Figure 2.8 illustrates extraction recoveries of the two systems while Table 2.6 summarizes quantitative results for the 100% acetonitrile extraction solvent system.

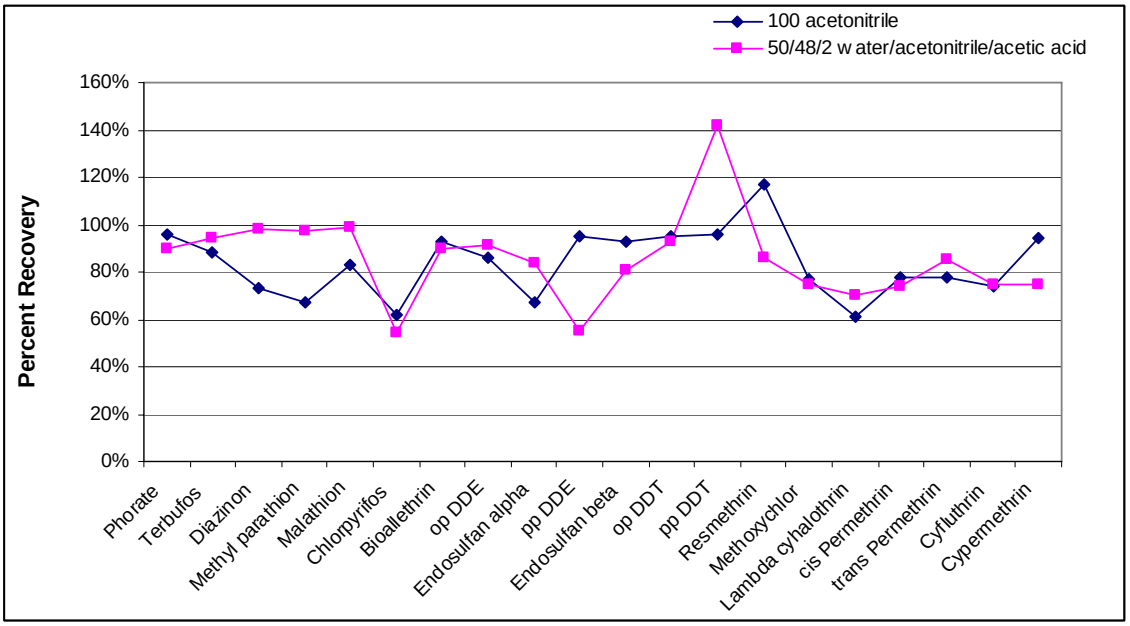


Figure 2.8 Percent Recovery of Target Analytes Comparing 100% Organic versus Aqueous-Organic ASE Extraction Solvents.

ANALYTE	PERCENT RECOVERY (+ / - SD)	ANALYTE	PERCENT RECOVERY (+ / - SD)
Phorate	96 (21)	Endo beta	93 (5)
Terbufos	88 (10)	opDDT	95 (8)
Diazinon	73 (9)	ppDDT	96 (13)
Methyl parathion	67 (15)	Resmethrin	117 (11)
Malathion	83 (14)	Methoxychlor	77 (12)
Chlorpyrifos	62 (19)	Lambda cyhalothrin	61 (6)
Bioallethrin	93 (14)	cis Permethrin	78 (7)
opDDE	86 (9)	trans Permethrin	78 (7)
Endo alpha	67 (8)	Cyfluthrin	74 (9)
ppDDE	95 (9)	Cypermethrin	94 (19)

Table 2.6 Percent Recovery for Target Analytes with 100% Acetonitrile ASE Extraction Solvent.

Results were comparable for both extraction solvents which was surprising because past studies have indicated higher recoveries with the addition of water to extraction solvents for soil and non-ionic pesticide extraction (Celi, Gennari et al. 1997; Dean 1999). Although recoveries were sufficient and evaporation times were greatly reduced, final samples were extremely dirty that resulted in poor chromatography. There were consistent chromatographic problems such as elution of many additional unknown peaks, peak shape deformities, loss of signal and intensity, and increase in background occurred after ~ five injections as demonstrated by methyl parathion and lambda cyhalothrin in Figure 2.9 and 2.10 respectively and is also indicated by high standard deviation shown in Table 2.6.

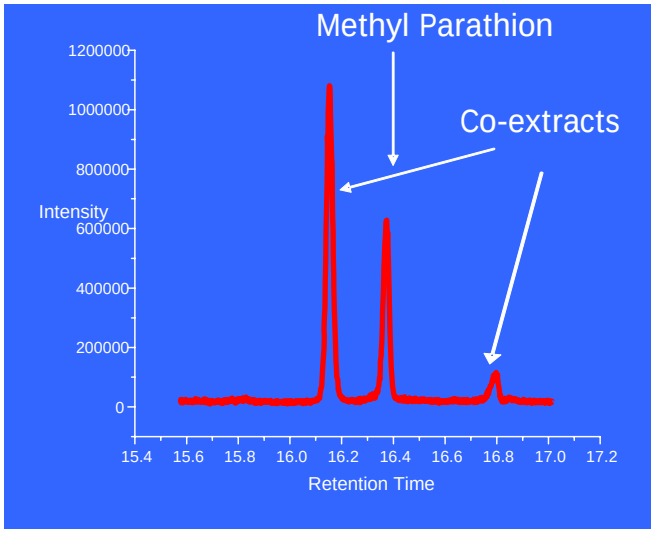


Figure 2.9 Methyl Parathion Co-Extracting Interferences.

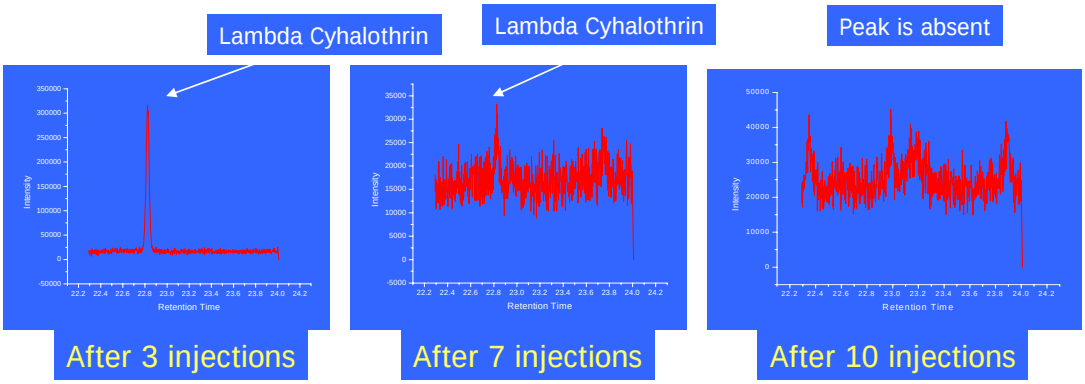


Figure 2.10 Loss of Lambda Cyhalothrin Signal after Multiple Injections.

Therefore, this particular method was deemed too problematic for selective and precise measurement. This was expected because ASE extraction is a rigorous extraction process at high pressures (1500psi or about 100 atm) releasing not only analytes of interest but many interfering materials from the soil as well, a common problem with exhaustive ASE environmental extractions (Steven M. Pyle 1997; Chuang, Hart et al. 2001; Pang, Liu et al. 2006; Zhao, Wang et al. 2006). In order to remove the interferences from the ASE extract and reduce evaporation times while retaining high recoveries, several selective

secondary-clean up steps were investigated to pre-concentrate the ASE extract before analysis.

Secondary Clean-Up Methods

Three different secondary clean-up methods were investigated and compared to obtain cleaner extracts and therefore higher quality method quantitative measurement: solid phase extraction (SPE), and liquid-liquid extraction (LLE) using traditional shake extraction and cartridge assisted extraction (see Figure 2.11 below).

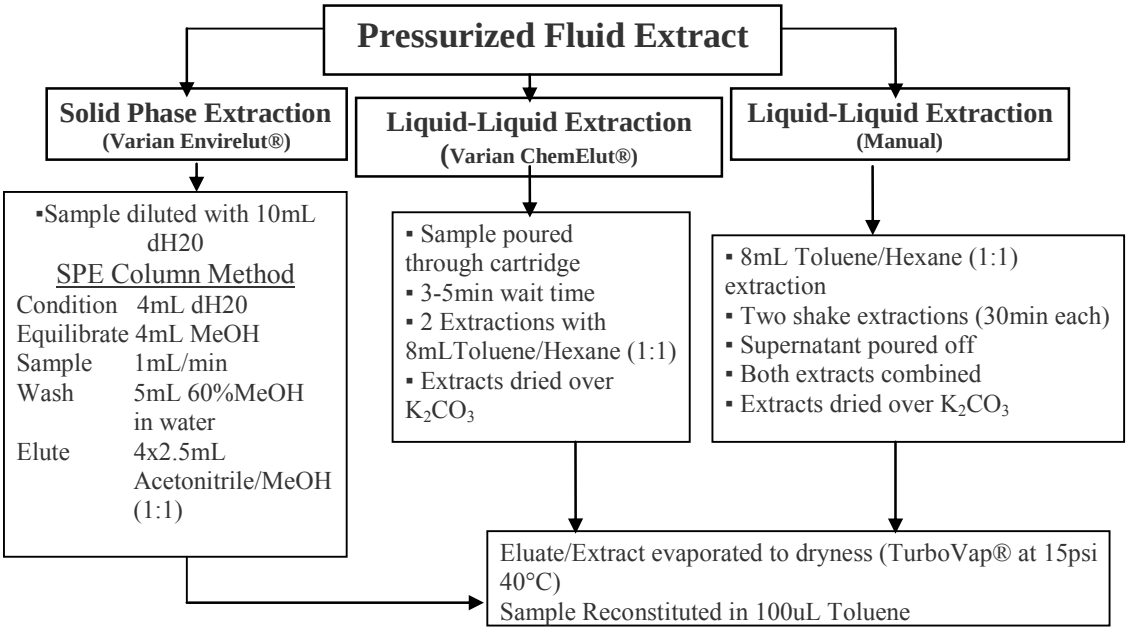


Figure 2.11 Three Separate Clean-Up Schemes of ASE Soil Extracts.

Solid Phase Extraction Optimization

Three key parameters of solid phase extraction methodology were investigated for optimization for the target analytes. Multiple cartridge sorbents, both silica based and polymeric based, were selected to investigate highest analyte retention. Various wash

solvents and elution solvents were also investigated to remove interferences and elute desired analytes from the cartridge respectively. All optimization parameters were carried out using spiked solvent samples (50/48/2 water/acetonitrile/acetic acid) first to focus on target analyte retention without interfering matrix effects. After optimization with spiked solvent samples, the chosen procedures were implemented using ASE extracted soil samples. Three different SPE cartridges were evaluated to remove many of the ASE co-extracting interferences and maintain high recoveries: Varian Nexus, Envirelut and Strata C18E (end-capped), details shown in Table 2.7.

Manufacturer	Sorbent Phase	Bonded Functional Group	Endcapped	Carbon Loading	Notes
Varian	Envirelut	Trifunctional Octadecyl/Silica based	No	18%	
	NEXUS	Mixed Mode Copolymer	No	0%	Actual bonding material held under proprietary notice; conditioning step not required
Strata	C18E	Trifunctional Octadecyl/Silica based	Yes	17%	

Table 2.7 SPE Sorbent Specifications.

Silica cartridges are the most commonly used sorbents for SPE (Simpson 2000). Large, bound octadecyl (C18) groups are used to modify silica to give the silica sorbent selective hydrophobic properties (Simpson 2000). Thus, in aqueous environments, C18 cartridges will retain hydrophobic analytes similar to the target analytes for this research. Endcapped silanols, one option for silica-based SPE, helps to prevent secondary interactions such as retention of polar, functional groups that can interfere with SPE (Simpson 2000). However, free silanols will still exist as the endcapping functionality is never 100% thorough (Simpson 2000). As an alternative to polar endcapping, polymeric sorbents are designed to minimize secondary interactions that are common with silica-based sorbents. Polymeric sorbents are made from organic materials (ex. styrene cross-

linked with divinylbenzene) that does not require pre-conditioning and can withstand extreme pH environments (Simpson 2000). Differences in recoveries were observed using the 3 SPE types and are summarized in Table 2.8 below.

Analyte	SPE CARTRIDGE Percent Recovery (+/-SD) (*n=12*)			
	Strata C18E[a]	Nexus[b]	Envirelut[c]	Envirelut[d]
Phorate	17(5)	13(3)	40 (4)	72 (3)
Terbufos	20 (5)	13 (2)	45 (6)	63 (1)
Diazinon	18 (3)	7 (1)	27 (3)	71 (4)
Methyl parathion	25 (8)	16 (1)	24 (2)	61 (3)
Malathion	25 (3)	25 (4)	25 (7)	117 (5)
Chlorpyrifos	34 (5)	29 (5)	77 (10)	98 (4)
*op*DDE	71 (7)	38 (4)	59 (11)	76 (13)
*pp*DDE	74 (7)	32 (6)	39 (8)	60 (8)
*op*DDT	66 (5)	61 (12)	69 (11)	100 (8)
*pp*DDT	70 (5)	82 (11)	139 (10)	100 (4)
Endosulfan *alpha*	73 (5)	62 (10)	102 (12)	101 (6)
Endosulfan *beta*	55 (5)	24 (2)	36 (5)	82 (5)
Methoxychlor	52 (5)	35 (7)	52 (2)	97 (1)
Bioallethrin	39 (5)	5 (3)	63 (3)	76 (6)
Resmethrin	2 (1)	25 (3)	46 (5)	23 (4)
Lambda cyhalothrin	30 (7)	55 (1)	97 (7)	94 (1)
cis Permethrin	61 (2)	69 (6)	78 (5)	73 (4)
trans Permethrin	67 (4)	73 (5)	85 (4)	73 (3)
Cyfluthrin	37 (3)	62 (1)	85 (11)	101 (5)
Cypermethrin	40 (3)	67 (3)	80 (10)	84 (7)

[a] 200mg sorbent bed
[b] 60mg sorbent bed
[c] 500mg sorbent bed
[d] 10mL deionized water + sample before load

Table 2.8 Percent Recovery for Target Analytes with Various SPE Clean-Ups.

SPE cartridges were preconditioned with 4 mL deionized water and equilibrated with 4 mL methanol (except NEXUS-no conditioning step required). Samples were then eluted with acetonitrile/methanol (1:1) four times each with 2.5mL each time. Samples were then dried over a few grains of potassium carbonate. The supernatants were poured off into new centrifuge tubes and evaporated to dryness using a Turbovap evaporator at 40°C

and 15psi. Samples were reconstituted with 100µL of toluene and transferred to autosampler vials for instrumental analysis.

Overall, the Envirelut cartridge gave the best recoveries. Some of the low recoveries were thought to be due to analytes possibly breaking through the sorbent bed with the loading solvent (deionized water/acetonitrile/acetic acid-50/48/2). This problem was further investigated by increasing the aqueous content to increase polarity of the ASE extract and maximize retention capacity of the C18 cartridges for target non-polar analytes prior to the loading step of the SPE process. The addition of deionized water improved recoveries for many of the analytes as seen in the last column of Table 2.8.

The Envirelut cartridges were chosen for further evaluation and optimized for wash solvent selection and elution solvent selection. The ideal wash solvent will be one that removes matrix interferences without eluting target analytes. The wash step was investigated by spiking 2mL deionized water and loading them after conditioning and equilibrating of the Envirelut cartridge. A gradient of methanol in water (0, 10, 20…-100%) was applied as the wash solvent. Samples were eluted with acetonitrile in methanol (1:1). Figure 2.12 below shows the trend for analyte retention based on % methanol in the wash solvent.

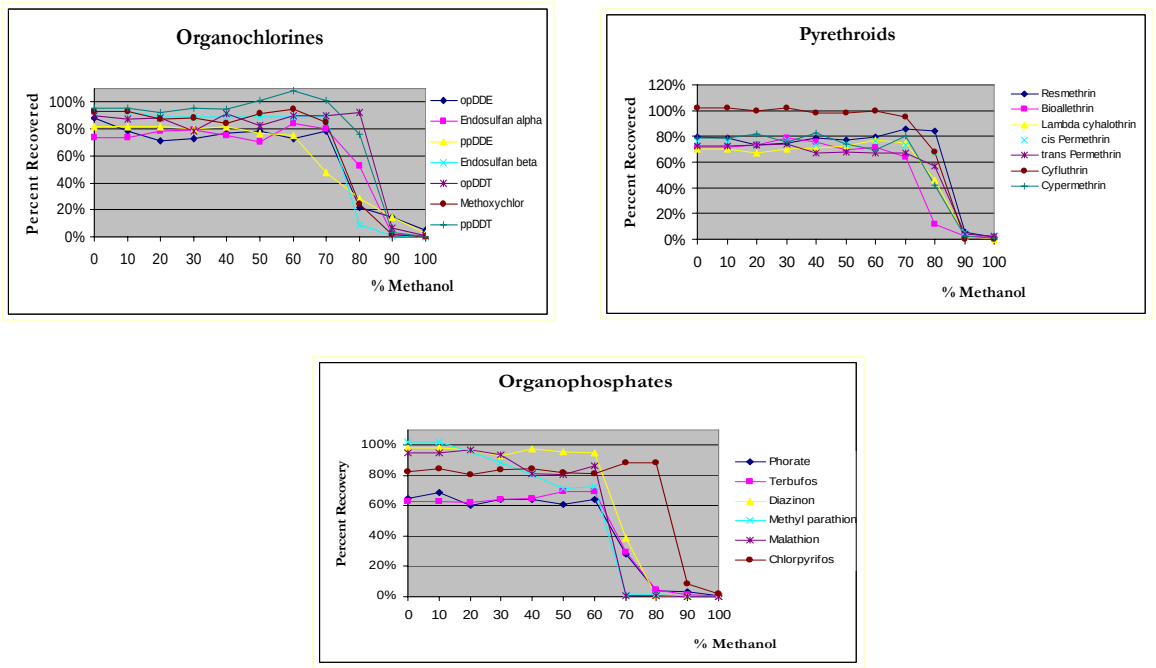


Figure 2.12 SPE Wash Break-Thru Analyses Shown by Pesticide Class.

The pyrethroids and organochlorines follow a similar pattern with most analytes beginning to wash off the column above 70% methanol in water. However, the organophosphates begin washing off analytes above 60% methanol indicating slightly higher polarity associated with these compounds. Overall, a 60% methanol in water wash was chosen as the optimum wash solvent. To optimize the elution step, nine different solvents were tested in decreasing polarity to determine which solvent(s) gave the best overall recoveries.

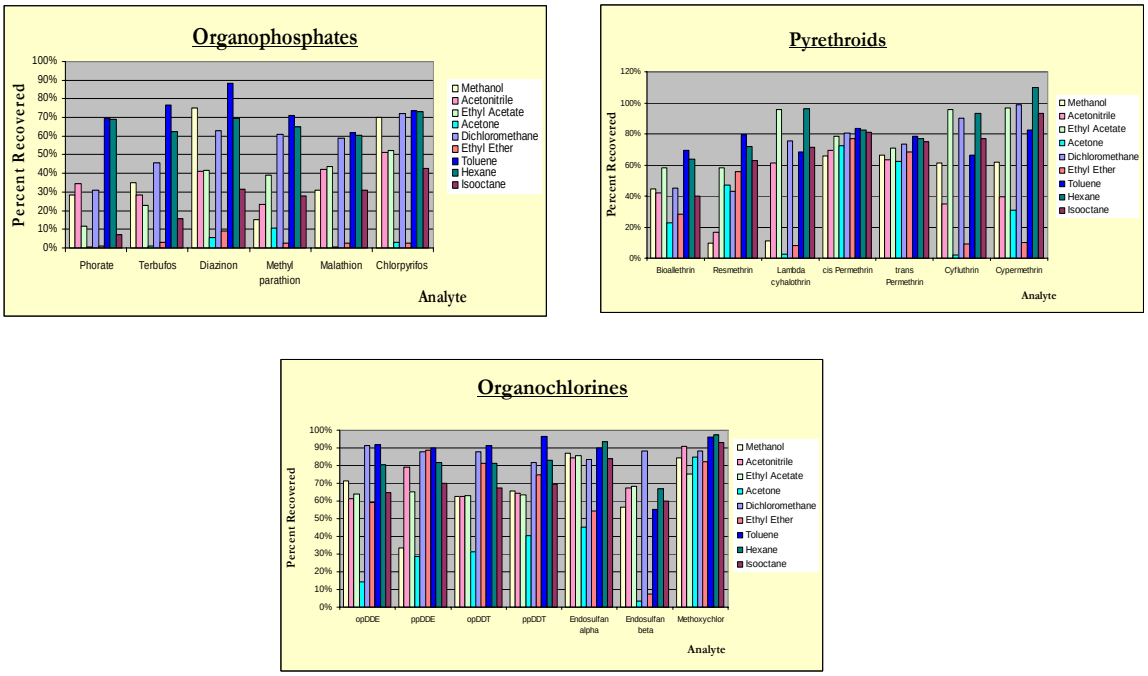


Figure 2.13 SPE Elution Step Analyses Separated by Pesticide Class.

Overall the best recoveries were obtained with toluene and hexane as elution solvents. This is most likely due to the nonpolar characteristic of most of the analytes. Toluene may help not only recover nonpolar analytes but aromatic analytes as well. However, using toluene and hexane together (1:1) as the elution solvent with the SPE clean-up presented additional challenges. Specifically, the chromatography was compromised showing loss of signal intensity, elevated background levels and distorted peak shapes after only few injections.

For cyfluthrin and cypermethrin, the peak intensity is greatly reduced after 10 injections and background increases dramatically thereafter resulting in poor quantitation as shown in Figure 2.14. The peak for methyl parathion is deformed by co-extracting interferences

after 12 injections with the appearance of a shouldering peak appearing after 15 injections (Figure 2.14).

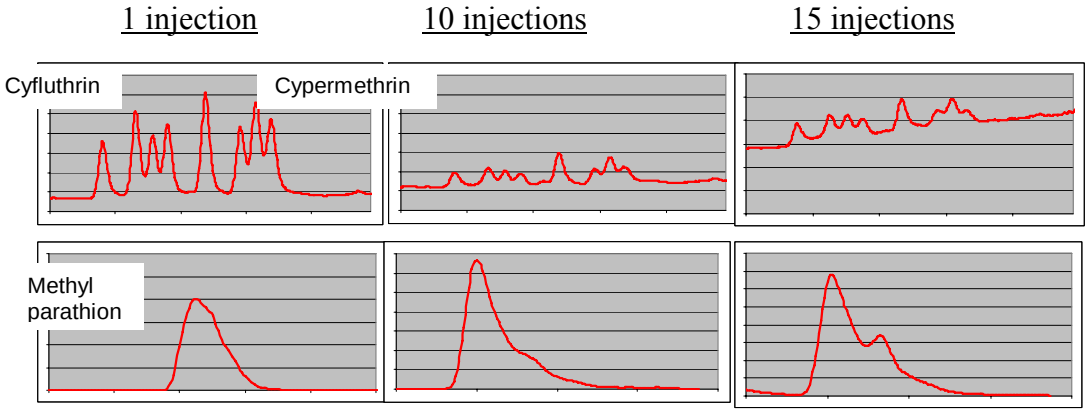


Figure 2.14 Chromatographic Interferences with Cyfluthrin, Cypermethrin and Methyl Paration with SPE Clean-up.

Considering that there were no matrix effects or ASE extraction effects contributing to the chromatographic interferences because these experiments were carried out with spiked solvent only, it was concluded that the contamination was coming from the only other possible source, the SPE cartridge itself. The chromatographic problems disappeared with less nonpolar solvents used to elute the analytes of interest. Therefore it is highly likely that using strong nonpolar solvents, such as toluene or hexane or the combination of both, compromises the integrity of the sorbent bed and dislodges parts of the sorbent and/or cartridge, possibly plasticizers or phthalates. Acetonitrile and methanol (1:1) as the elution solvent was consequently chosen as the elution solvent as this mixture still gave good recoveries (20-120%, with most analytes between 60-100%) and maintained chromatographic and instrumental integrity (Figure 2.15).

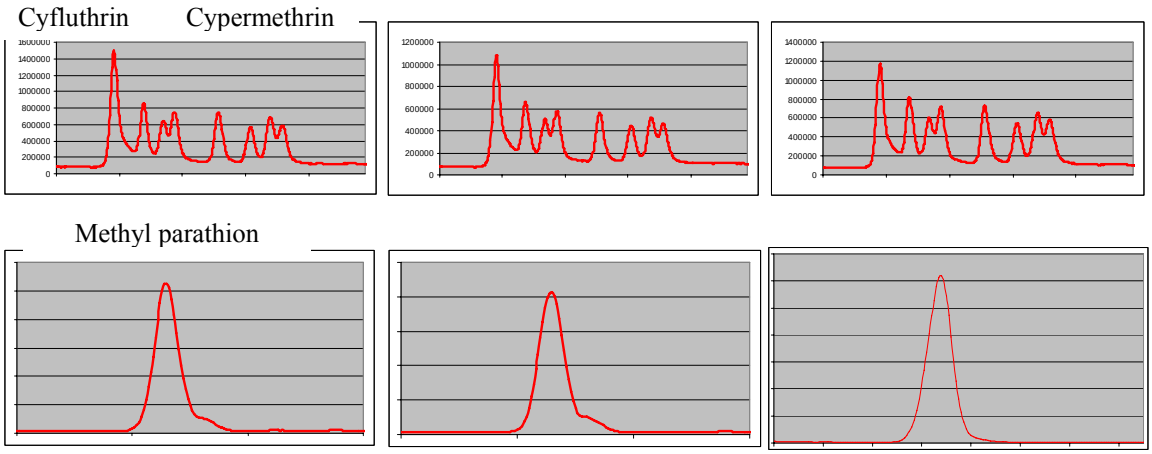


Figure 2.15 Chromatographic Improvement with Cyfluthrin, Cypermethrin and Methyl Parathion with Different Elution Solvent for SPE

Liquid-Liquid Extraction Optimization

The goal for the liquid-liquid extraction method investigation was to compare gravity solvent flow extraction with the cartridge-immobilized Hydromatrix and a more traditional LLE technique, high-power shake-flask method. Two parameters were investigated with liquid-liquid extraction techniques (both cartridge and shake-flask methods): solvent selection and volume of extraction solvent. All liquid-liquid extraction analyses were performed using spiked 20mL ASE extraction solvent (50/48/2 water/acetonitrile/acetic acid) samples to preclude matrix interferences and observe analyte behavior only. After optimization for these methods, they were applied to soil ASE extracts to confirm results. Each sample was manually extracted with the appropriate extraction solvent by shaking (Glas-Col, Terre Haute, IN) for 30 minutes. The extraction was performed twice. Both extracts were combined and dried over potassium carbonate. The supernatants were poured off into new centrifuge tubes and

evaporated to dryness at 40°C and 15psi. Samples were reconstituted with 100 µL of toluene and transferred to autosampler vials for instrumental analysis.

Sorbent-immobilized extraction cartridges from Varian were analyzed as an alternate liquid-liquid extraction method. The samples were applied to the 20 mL extraction cartridges and allowed to sit for 5 minutes to evenly distribute sample into the sorbent. The samples were then eluted with appropriate extraction solvent allowing all of the solvent to seep through the cartridge before repeating the extraction. Samples eluted no faster than 1mL/minute. The eluates were collected in centrifuge tubes and samples were dried over potassium carbonate. Supernatants were poured off into new centrifuge tubes and then evaporated to dryness at 40°C and 15psi. Samples were reconstituted with 100 µL of toluene and transferred to autosampler vials for instrumental analysis

Six solvents were investigated to determine highest recoveries, restricting the investigation to nonpolar solvents (more polar solvents, e.g., acetonitrile, methanol, etc., were excluded) to promote complete phase separation between ASE extract and secondary organic extract. Results are shown in Figure 2.16. All extractions were performed twice with 10mL solvent each ($n=5$).

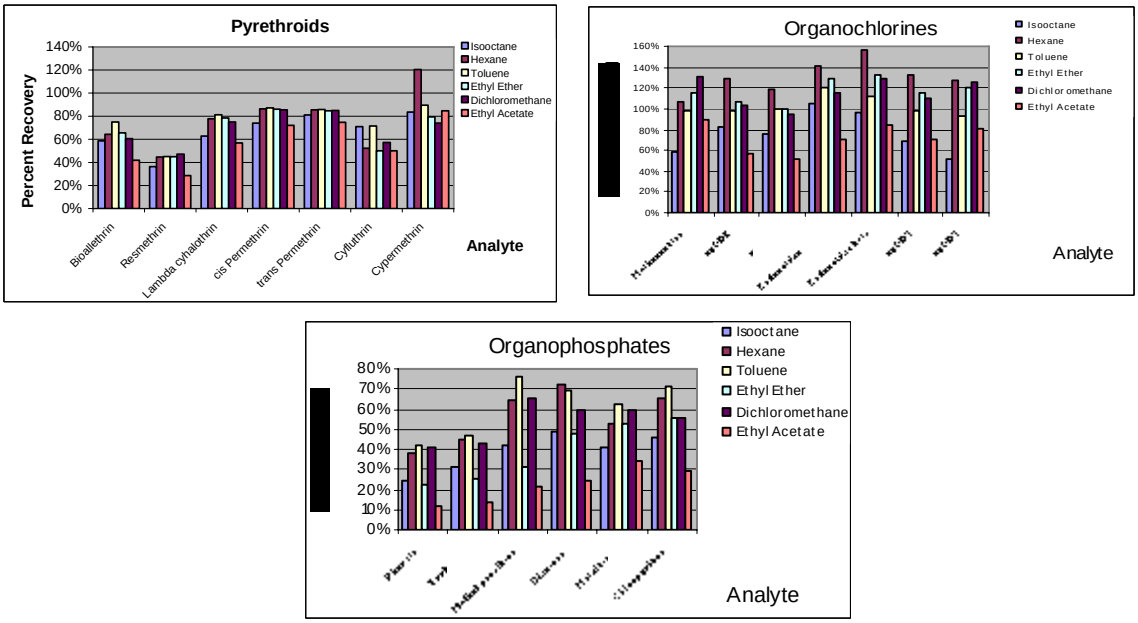


Figure 2.16 Percent Recovery for Target Analytes with LLE using Various Solvents Separated by Pesticide Class.

Similar to SPE, organophosphates and pyrethroids showed the highest recoveries using hexane and toluene as elution solvents. The organochlorines can be eluted with any of the solvents selected although ethyl acetate and isooctane both gave lower recoveries. Toluene and hexane were chosen as elution solvents for the manual liquid-liquid extractions and then applied to the cartridge assisted liquid-liquid extraction method to determine if recoveries were comparable (Figure 2.17).

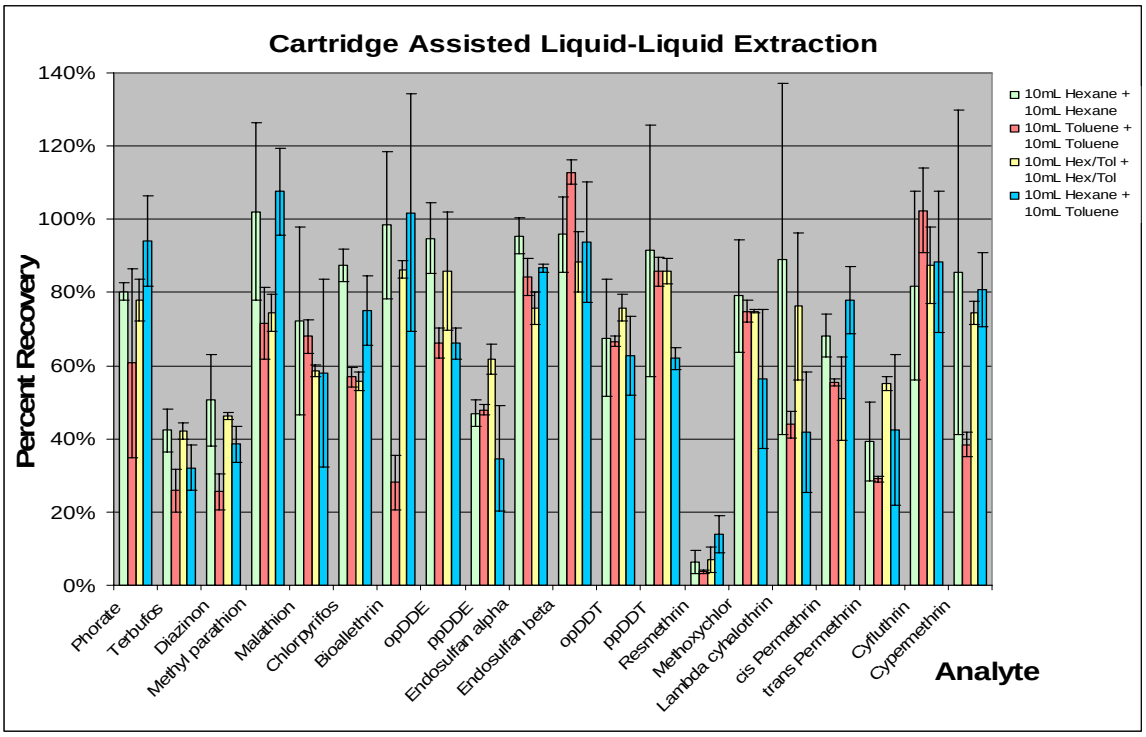


Figure 2.17 Percent Recovery of Target Analytes with Cartridge-Assisted LLE using Various Solvents.

Several solvent systems were selected for the cartridge extractions (Each extraction with 10mL solvent). Figure 2.17 shows that two of the solvent systems (10mL hexane followed by 10mL toluene and 10mLof 1:1 hexane/toluene followed by 10mL of 1:1 hexane/toluene) give higher recoveries than by extracting with hexane or toluene alone. This indicates that binary solvent extraction may be more productive due to both a nonpolar alkyl chain component (hexane) and nonpolar aromatic component (toluene), which are both physical characteristics in common with the analytes of interest. The binary solvent mix (1:1 toluene/hexane) was chosen as the extraction solvent as it gave higher recoveries with lower standard deviation than the binary solvent of hexane extraction followed by toluene extraction.

Extraction volume was another variable of the method that was optimized. For both the manual liquid-liquid extraction method and the cartridge assisted liquid-liquid extraction method, two extractions (at 30 minutes each for manual LLE) of 4mL, 6mL, 8mL, and 10mL of Toluene/Hexane (1:1) were investigated as seen in Figure 2.18.

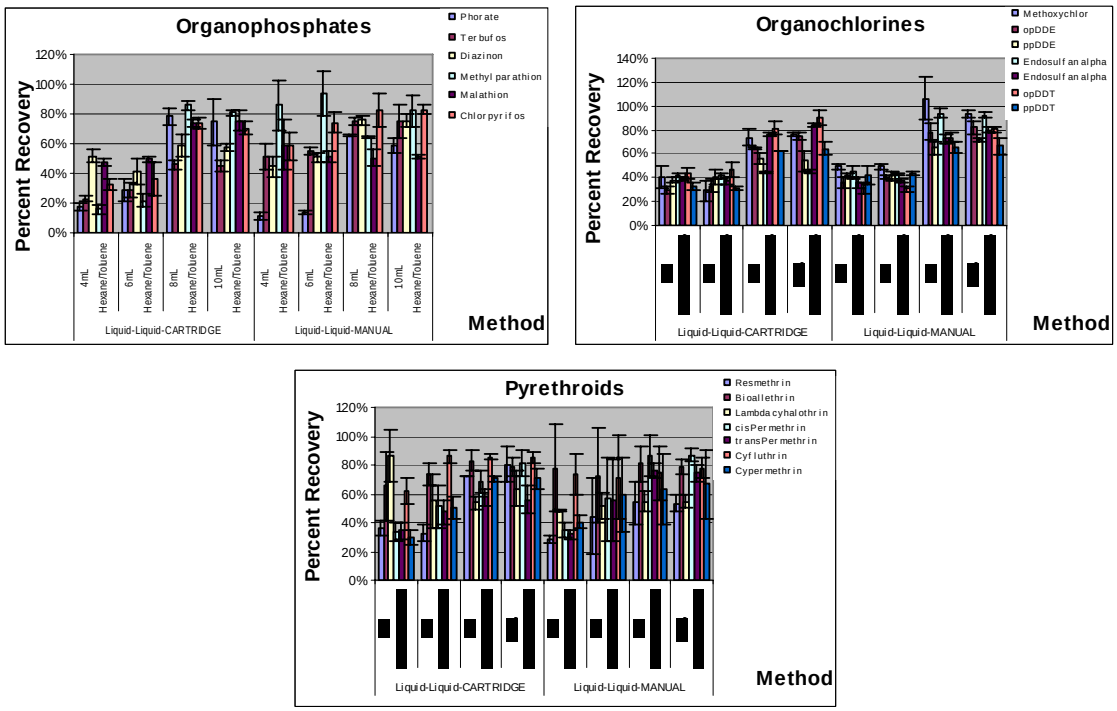


Figure 2.18 Percent Recovery for Target Analytes Comparing Extraction Volumes for LLE.

Figure 2.18 illustrates two important results of the liquid-liquid extraction methods. First, it compares how solvent volume affects extraction of the analytes. Secondly, it compares differences in recovery between cartridge-assisted liquid-liquid extraction and conventional manual liquid-liquid extraction. For example, there was a distinct difference for organophosphate extraction between the two methods. The recoveries for organophosphates doubled using ≥8mLs extraction solvent with the cartridge-assisted extraction compared to the manual method where recoveries were similar for all extraction volumes (excluding phorate which behaved similarly for both methods). The

organochlorines are in contrast to the organophosphates. There is a bimodal type distribution for this class compounds with both methods showing similar results (both methods give better recoveries for ≥8mL extraction solvent). Individual pyrethroids behaved dissimilar to each other and did not follow a clear pattern. For example, cypermethrin showed a clear pattern of increasing percent recovery as the solvent volume increases for both methods. However, bioallethrin and cyfluthrin both are relatively constant using any volume of solvent for either method. Overall, using ≥8ml extraction solvent volumes gives better recoveries than using less volume.

Method Validation

Total analyte recovery and detection limits were calculated for all three extraction methods (ASE followed by either: SPE, traditional LLE or cartridge-assisted LLE). Quality control characterization and linearity were additionally determined for the ASE-manual LLE method as this method gave the best overall analyte recoveries and detection limits.

Analyte Recovery

Analyte recoveries for all three extraction methods were determined at low and high concentrations (25ng/g and 100ng/g respectively) for each analyte. Soil samples (*n=10*) were spiked at these concentrations, extracted using ASE and normalized to "100%" recovery samples spiked after extraction and before instrumental analysis. A summary of percentage recovered with respect to the 100% samples are summarized by method and pesticide class below in Figure 2.19.

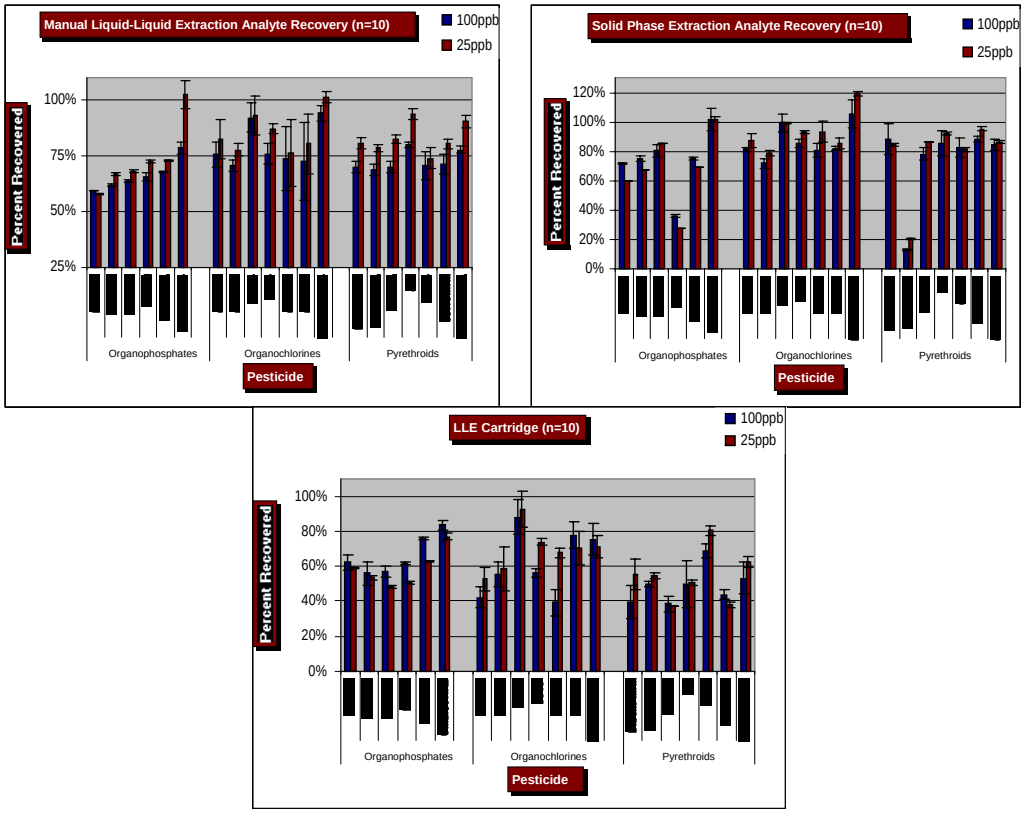


Figure 2.19 Percent Recovery for Target Analytes at 2 Levels Comparing 3 Extraction Methods.

Analyte concentration did not affect recovery as both concentrations had similar results for all methods indicating validity over a range of concentrations that are used for the standard curve. Overall, clean-up of the ASE extract with solid phase extraction gives the highest recoveries except for resmethrin and methyl parathion which were both recovered at less than 40%. This could be a factor of a more polar nature (low log D value and higher water solubility) associated with methyl parathion that may cause breakthrough with the load step of the SPE. Resmethrin has been somewhat problematic for all the analysis with lower recoveries, although with the manual clean-up, it gives ~75% recovery. The low recoveries for resmethrin have been observed before and are

thought to be due to poor thermal stability and thus unsuitability for GC analysis (Pang, Liu et al. 2006; Woudneh and Oros 2006). Recoveries for manual clean-up were slightly lower with most analytes (17/20) averaging 75% recoveries for both 25 and 100ppb. Recoveries for the cartridge clean-up method were the lowest with 14/20 analytes averaging 60% recovery for both 25 and 100ppb samples.

Detection Limits

There are many different methods for determining detection limits of a method. For this paper, an experimentally derived calculation was used to determine detection limits which was then verified by injecting samples prepared at the derived detection limit to observe peak intensity relative to background signal. Detection limits were obtained for each analyte by each of the three extraction methods by using the Taylor method (Taylor 1987). The Taylor method calculates the regression of the standard deviation of the standards at their concentrations. The intercept therefore, represents the standard deviation of the zero concentration. Typically, three times this value, $3*S_0$, is used to estimate limit of detection. All Taylor-derived detection limits were verified by injection of the samples prepared at calculated concentration to ensure discernible peaks separate from background which also met the 3 times signal to noise ratio rule (this was calculated by Xcalibur, Thermo acquisition software).

Initially, instrument detection limits (IDL) were determined to isolate analyte and instrument from any possible matrix effects. Sample aliquots of 100uL of toluene were spiked with native and label compounds with varying concentrations (1 ng/g, 5ng/g,

10ng/g, 20 ng/g, 50 ng/g, 100 ng/g). Method detection limits (MDL) were then determined with soil samples with complete extraction at the same concentrations for instrument detection limits. Detection limits determined for all analytes are summarized in Table 2.9 below.

Detection Limits

	Instrument	Cartridge LLE	Manual LLE	SPE
alpha-Endosulfan	1.33	24.20	6.80	65.40
beta-Endosulfan	1.43	8.30	0.60	6.10
Bioallethrin	0.66	8.00	6.80	4.40
Chlorpyrifos	1.20	7.00	7.30	3.00
cis-Permethrin	4.98	1.00	2.40	3.30
Cyfluthrin	0.33	38.70	4.30	9.60
Cypermethrin	1.90	31.80	2.20	2.60
Diazinon	0.82	4.20	5.00	16.10
Lambda Cyhalothrin	3.87	11.00	13.70	2.10
Malathion	1.09	12.30	1.30	0.60
Methoxychlor	1.54	4.80	3.10	2.40
Methyl Parathion	1.44	4.10	2.70	0.10
op-DDE	0.82	23.40	11.50	3.30
op-DDT	0.52	14.10	13.40	4.80
Phorate	0.77	4.30	2.70	9.80
pp-DDE	1.46	16.80	7.40	2.00
pp-DDT	0.79	8.50	10.30	19.80
Resmethrin	2.30	9.00	4.40	59.70
Terbufos	0.90	7.00	2.00	4.50
trans-Permethrin	4.17	7.30	1.30	7.20

Table 2.9 Target Analyte Instrument and Method Detection Limits.

Instrument detection limits were found to be in the high parts-per-trillion (ppt) to low ppb range (0.33-4.98 ppb) and method detection limits were found mostly in the low ppb range from 0.6-13.7 ppb (manual LLE), 1-38.7 ppb (cartridge LLE), and 0.1-65.4 ppb (SPE).

Interestingly, several analytes were found to have method detection limits below the instrument detection limit (*beta*-endosulfan, *cis*-permethrin, *trans*-permethrin, lambda cyhalothrin, malathion, methyl parathion). This is most likely due to matrix effects resulting in signal enhancement which leads to more intense peaks at lower concentrations. Although matrix effects are not as common with GC-MS methods as they are with LC-MS methods, they are still documented in several applications (Garrido-Frenich, Romero-Gonzalez et al. 2006; Larreta, Vallejo et al. 2006; Sanchez-Brunete, Miguel et al. 2006). Overall, the limits of detection found for manual liquid-liquid extraction clean-up are the lowest. The high detection limits found for resmethrin with the SPE clean-up are congruent with the low recoveries suggesting that this method of clean-up is inefficient for extraction of this analyte. For methyl parathion, the calculated Talyor detection limit (100 ppt) for the SPE method did not match the injected concentration. No peak was observed higher than the background at 100ppt. In fact, only at a level 10* this amount (1ppb) is there an observable peak of 3*SN as shown in Figure 2.20. For methyl parathion, the Taylor method is somewhat liberal probably due to good precision at lower standards which is the prime determinant for detection limit calculation.

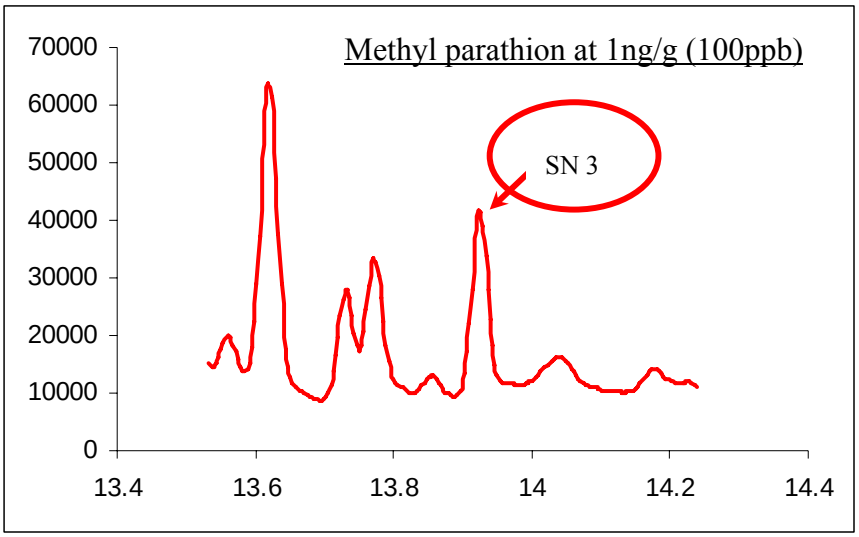


Figure 2.20 SN of Methyl Parathion at 100ppb with SPE Clean-Up.

This is opposite for diazinon which has a detection limit of 16.1 ppb found using the Taylor method with the SPE clean-up. Figure 2.21 shows an injection at 5ppb with a clear peak and SN ratio of 5 which implies that the detection limit is lower even than 5ppb.

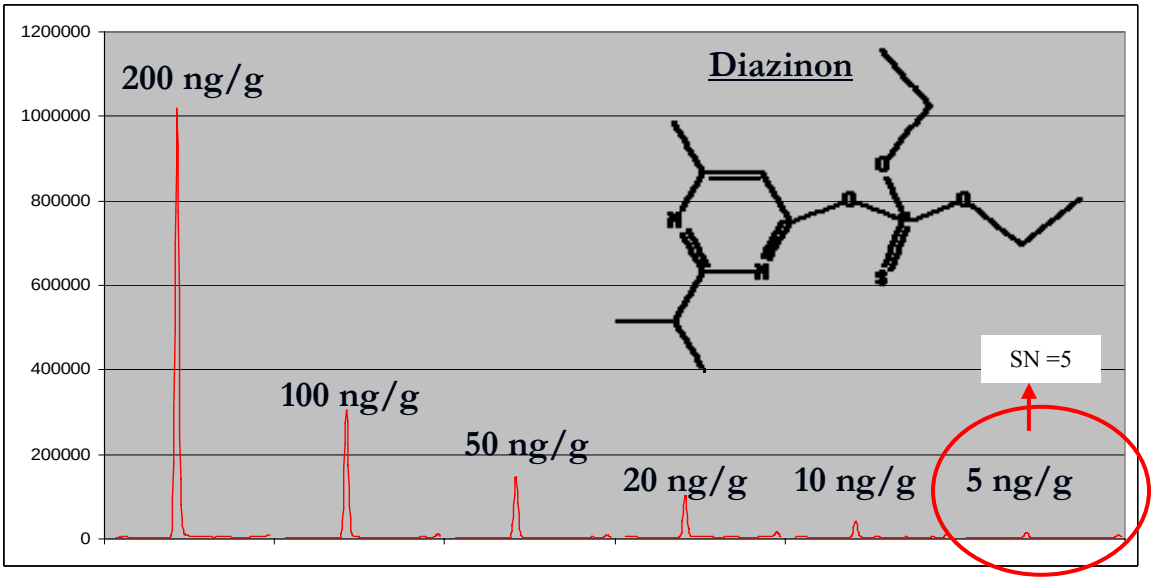


Figure 2.21 SN of Diazinon at 5ppb with SPE Clean-Up.

This implies that the detection limit found using the Taylor method is somewhat conservative for diazinon most likely due to poor precision of this analyte with this method at low concentrations. The differences observed for methyl parathion and diazinon show the importance of verifying calculated detection limits with observable results.

Precision and Accuracy

In terms of best overall recoveries and lowest detection limits, manual LLE second clean-up following ASE extraction was chosen for further consideration. Additional method validation parameters such as precision, accuracy and linearity were all determined of this method. Quality control characterization was used to assess the quality of the analytical method as defined by precision and accuracy of the method. Initially, two pools (low and high concentration) of QC standards were prepared using 50g of soil. Pools were spiked at 25ng pesticide/g soil and 50ng pesticide/g soil as QC-low and QC-high standards respectively. Spiking was accomplished by placing blank soil in a clean container, spiking the soil with appropriate standard concentration and adding sufficient acetonitrile to completely cover the soil. The container was then capped and mixed using a rotary mixer for five days to ensure homogeneity of the spiking. After the five days, the cap was removed to allow evaporation of the solvent. QC pools were then recapped and stored in a -20ºC freezer to prevent degradation until analysis. Multiple 1g aliquots ($n=6$) were taken from each pool for 4 days in succession to analyze precision within each day and between days as relative standard deviation (RSD). Samples were spiked

with internal standard and extracted using the ASE-manual LLE method with a single blank sample and complete calibration curve. Precision measurements are summarized below in Table 2.10.

	Within Day Variation (%)		Between Day Variation (%)	
	QC-Low (25 ng/g)	QC-High (100 ng/g)	QC-Low (25 ng/g)	QC-High (100 ng/g)
alpha -Endosulfan	6.0	5.0	26.0	14.0
beta- Endosulfan	12.0	10.0	52.0	31.0
Bioallethrin	8.0	13.0	25.0	21.0
cis -Permethrin	11.0	13.0	19.0	16.0
Cyfluthrin	16.0	14.0	57.0	19.0
Cypermethrin	11.0	11.0	30.0	15.0
Diazinon	11.0	14.0	35.0	29.0
Lambda Cyhalothrin	10.0	13.0	27.0	17.0
Malathion	10.0	16.0	35.0	22.0
Methoxychlor	11.0	7.0	14.0	13.0
Methyl Parathion	7.0	12.0	30.0	14.0
op -DDE	3.0	3.0	7.0	11.0
op -DDT	5.0	6.0	12.0	11.0
Phorate	27.0	25.0	51.0	51.0
pp- DDE	5.0	5.0	9.0	10.0
pp -DDT	8.0	7.0	13.0	12.0
Resmethrin	19.0	24.0	30.0	53.0
Terbufos	16.0	21.0	53.0	44.0
trans -Permethrin	11.0	14.0	20.0	16.0

Table 2.10 Within-Day and Between-Day Variation of ASE-Manual LLE Method.

The RSDs for the analytes within-day analysis are good (between 3-27%) considering there is not an internal standard for every analyte. The poor precision seen for phorate and terbufos may be a result of lack of corresponding label internal standard as well as lower recoveries associated with these compounds (~60%). Between-day variation was very poor with RSDs found between 7-57% as shown in Figure 2.22. After investigation into the cause of this problem, it was determined that all of the analytes had higher

calculated amounts on day 3 of the four day QC characterization which decreased overall

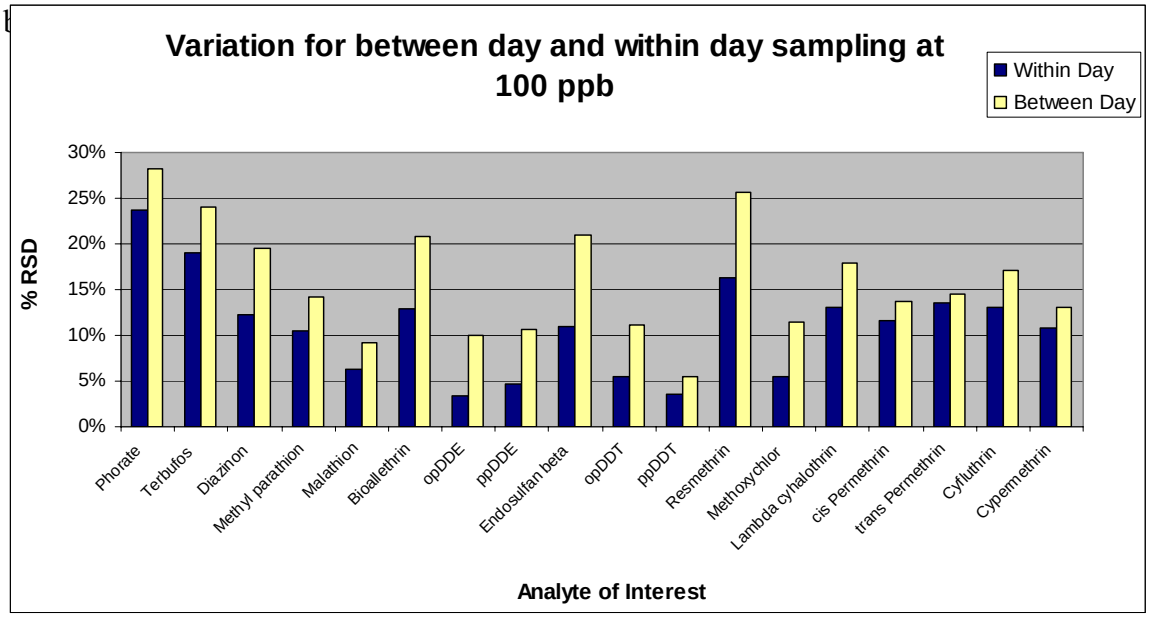


Figure 2.22 Within-Day and Between-Day Variation of Target Analytes at 100ppb with ASE-Manual LLE Method.

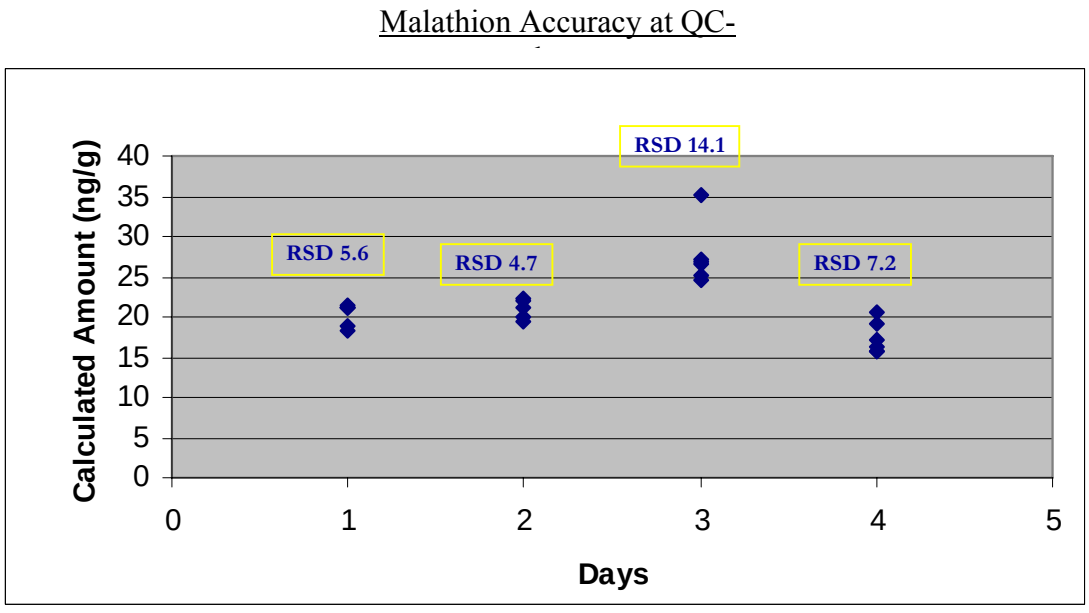


Figure 2.23 Accuracy of Malathion at 25ppb with ASE-Manual LLE Method.

Days 1, 2 and 4 yield relatively equivalent calculated amounts but day 3 calculated amounts are higher. An explanation for this may be due to less internal standard added that day resulting in a greater area ratio. Because internal standard is added manually, this may be corrected with automation to improve overall precision.

Accuracy of the analytical method measures the degree of agreement between the averaged calculated amounts derived from the calibration curve and the spiked concentration with values +/-15% deemed acceptable. As shown in Figure 2.24, resmethrin is accurate when averaged (average 24.1ng/g) but has relatively poor precision. Determination of average accuracy for all analytes of interest is shown in Table 2.11(*n=30)*.

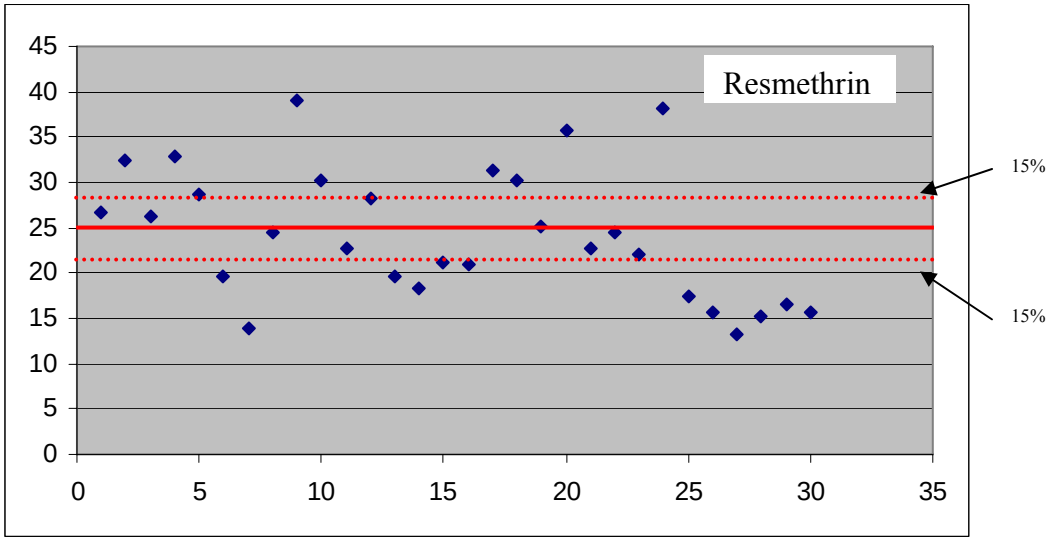


Figure 2.24 Accuracy and Precision of Resmethrin at 25ppb with ASE-Manual LLE Method.

	QCL (25ppb)	QCH (100ppb)
Phorate	22.4	79.8
Terbufos	28.2	94.4
Diazinon	28.0	106.2
Methyl parathion	24.0	95.0
Malathion	25.5	105.0
Bioallethrin	21.2	87.3
op-DDE	30.1	114.4
alpha-Endosulfan	20.8	104.1
pp-DDE	30.3	117.3
beta-Endosulfan	26.1	110.5
op-DDT	32.4	118.2
*pp-D*DT	27.0	113.0
Resmethrin	24.1	87.6
Methoxychlor	29.3	113.3
Lambda cyhalothrin	20.6	87.8
cis-Permethrin	19.4	89.6
trans-Permethrin	19.7	90.4
Cyfluthrin	21.8	78.3
Cypermethrin	19.7	88.3

Table 2.11 Average Accuracy (*n=30*) of Target Analytes with ASE-Manual LLE Method.

Unfortunately, many of the target analytes did not fall within +/-15% of the spiked value at the QC-low level (25ng/g) and thus would be considered unacceptable. In consequence, lower-end concentrations should be only assessed qualitatively rather than quantitatively. However, only phorate, *pp*DDE, and *op*DDT were +/-15% of the spiked QC-high value (100ng/g) indicating that this method is more valid for quantitative assessment at higher concentrations further away from the detection limits of the analytes.

Linearity

All regression correlation coefficients were calculated from linear fits. All analytes were highly linear with respect to the calibration curve as shown in Table 2.12. Figure 2.25 shows an example of the linearity of *cis* permethrin with a correlation coefficient of 0.99. The lower range of the calibration curve is still linear with a correlation coefficient of 0.95. Only phorate (0.9821) and terbufos (0.9892) had correlation coefficients less than 0.99. This may be a factor of the poor precision associated with these two analytes.

	R^2 of Calibration Curve
Phorate	0.9821
Terbufos	0.9892
Diazinon	0.9960
Methyl parathion	0.9983
Malathion	0.9923
Bioallethrin	0.9980
op-DDE	0.9995
alpha-Endosulfan	0.9938
pp-DDE	0.9995
beta-Endosulfan	0.9953
op-DDT	0.9969
*pp-D*DT	0.9996
Resmethrin	0.9906
Methoxychlor	0.9902
Lambda cyhalothrin	0.9993
cis-Permethrin	0.9974
trans-Permethrin	0.9984
Cyfluthrin	0.9985
Cypermethrin	0.9980

Table 2.12 Linearity of Target Analytes with ASE-Manual LLE Method.

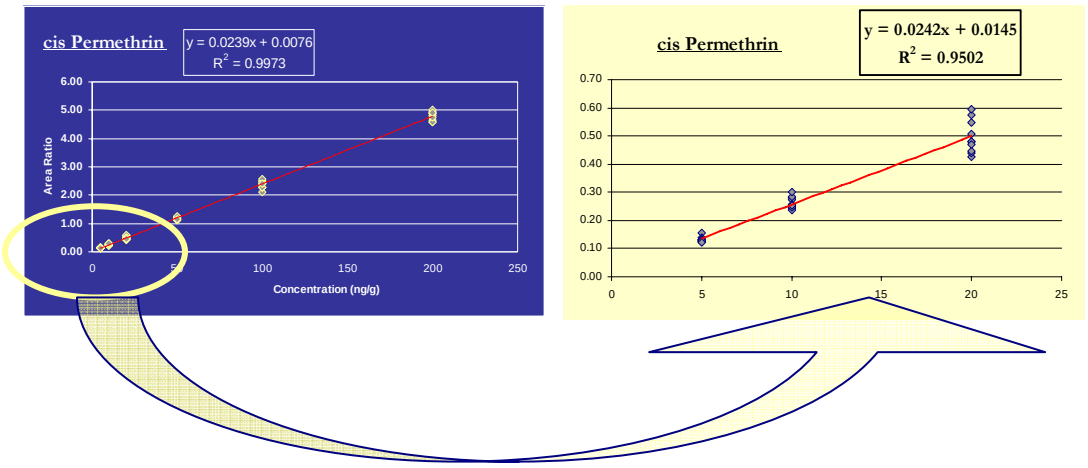


Figure 2.25 Linearity of *cis* Permethrin across Calibration Curve and Lower End of Curve (in yellow).

Chlorpyrifos

Chlorpyrifos had to be removed from the list of analytes of interest because of the presence of endogenous amounts of chlorpyrifos in sample soil. After analysis of calibration curves, chlorpyrifos did not establish linearity but at each concentration, would give similar amounts as shown in Figure 2.26.

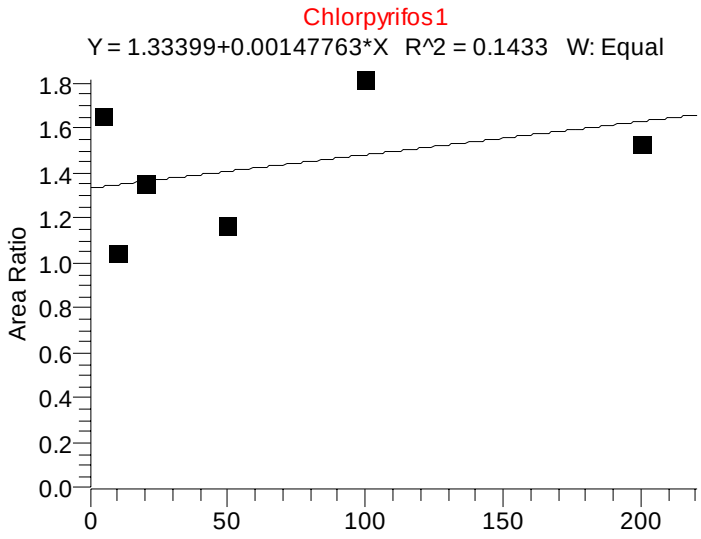


Figure 2.26 Lack of Linearity of Chlorpyrifos Due to Background Presence.

Several possibilities exist that could result in loss of linearity: possible internal standard (chlorypyrifos methyl) degradation during extraction and/or instrumentation or contamination of soil with chlorpyrifos. Injections were made with internal standard only (in solvent-without matrix) to determine if degradation occurred in the inlet, oven, etc. because these parameters are held at high temperatures. In addition, soil samples with internal standard only and blank soil samples (no internal standard or natives) were prepared mimicking sample preparation (ASE+ manual LLE). Evidence concluded that blank soil had high concentrations of endogenous chlorpyrifos present which is demonstrated in Figure 2.27.

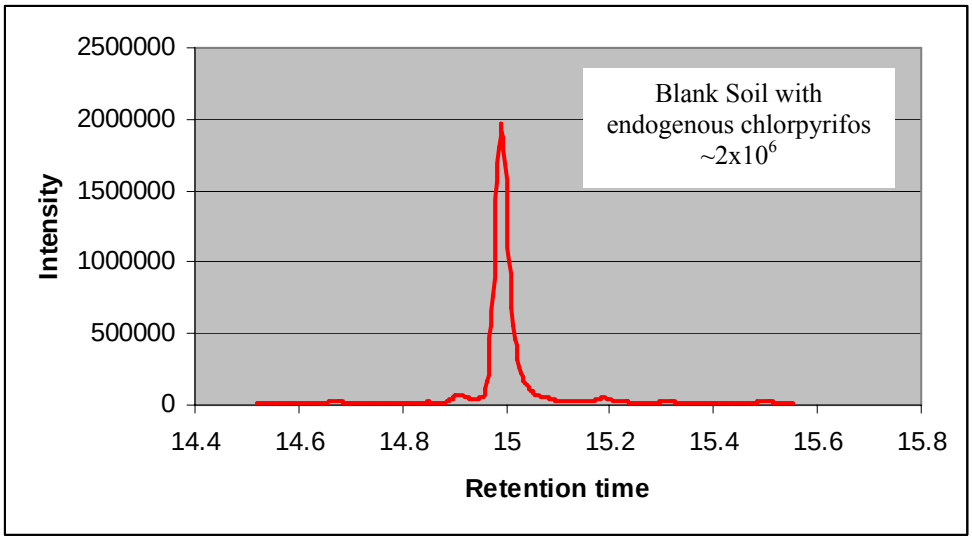


Figure 2.27 Blank Soil Sample with Endogenous Chlorpyrifos Present.

Conclusions

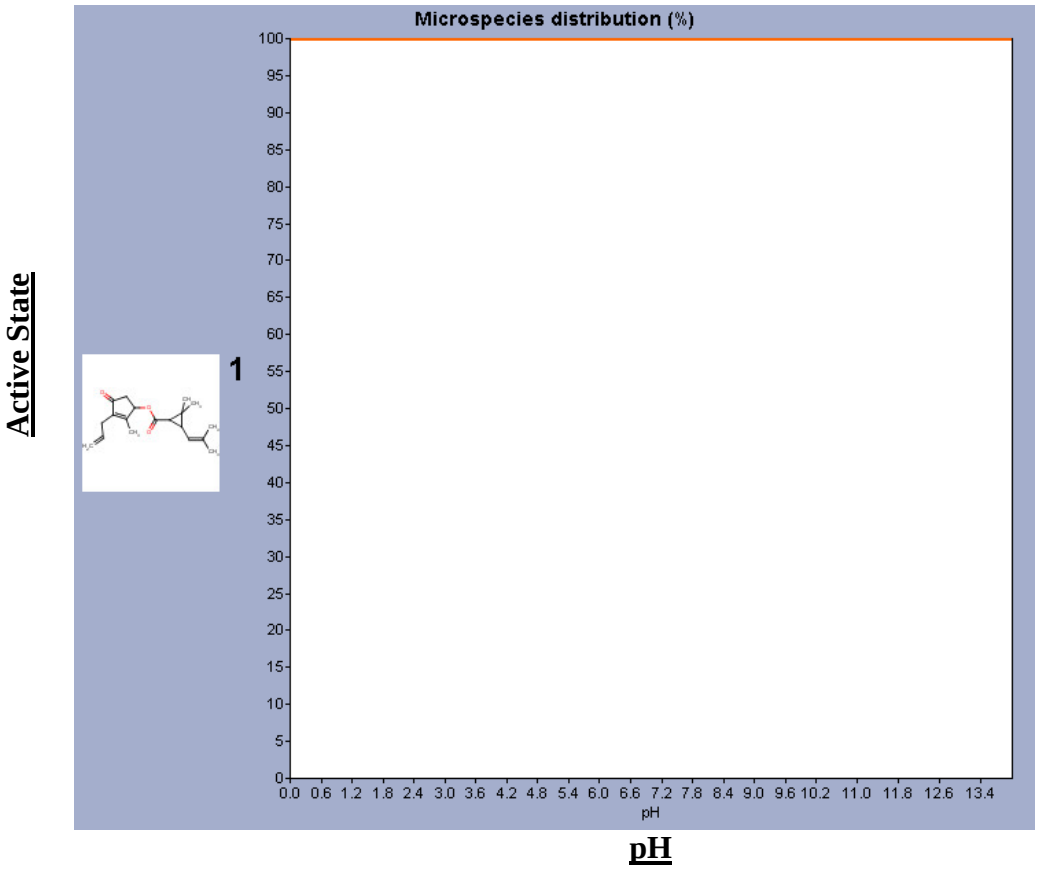
A mass spectrometry based-analytical method has been developed for the measurement of three classes of pesticides: organophosphates, pyrethroids and organochlorines in 1g soil. Three post-ASE extraction secondary clean-ups were evaluated in terms of their analyte recovery and detection limits. Overall, the manual-LLE clean-up method had the highest recoveries with the lowest detection limits. Upon further assessment of the total ASE, manual-LLE method with soil provided good within-day precision but poor between-day precision, a situation that can be corrected with automated standard/internal standard addition. This method also proved highly accurate at the higher end of the calibration curve (100ng/g) for all target analytes except for phorate, *pp*DDE, and *op*DDT which were within ~20% of the spiked concentration. Unfortunately, only the OP pesticides were within +/-15% of the spiked concentrations at the lower end (25ng/g). Only 3/6 pyrethroids and 2/7 organochlorines were within acceptable accuracy range of their spiked concentrations. Therefore, this method would be adequate for only qualitative assessment toward the lower end of the calibration curve. As stated earlier, upon re-evaluation of the precision measurements, it was determined that one day (day 3) had inconsistent results compared to the other days (days1,2,4). In consideration of this aberrant day, re-calculation of accuracy excluding day 3 yielded all target analytes within +/-15% of spiked concentration at both low and high ends (except for *op*DDT). Again, it was concluded that upon automation of standard handling for this method, QC characterization measurements would be improved.

It was determined that endogenous amounts of chlorpyrifos were present in blank soil used to for analytical method evaluation. Consequently, chlorpyrifos was removed from further consideration as it interfered with method evaluation parameters quantitation. This was unfortunate as the recent findings of adverse health effects and subsequent banning of chlorpyrifos has made it an extremely important OP to measure and quantify in environmental matrices. To correct this problem in the future, either the soil could be washed to remove endogenous chlorpyrifos or soil could be collected from other sources and evaluated for possible background amounts of chlorpyrifos.

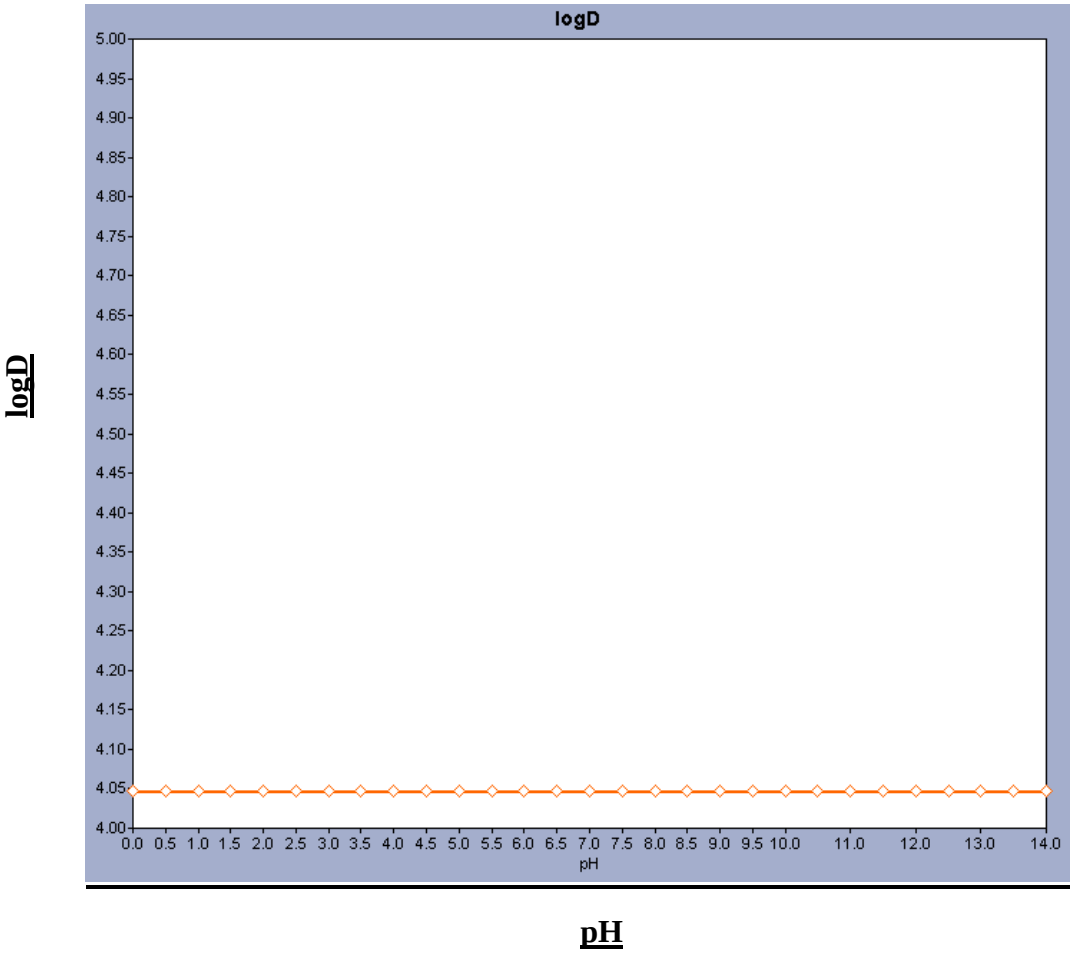
Appendix 2A

The following figures detail physiochemical details of target analytes (generated from ChemAxon© MarvinSketch version 1.4.6):

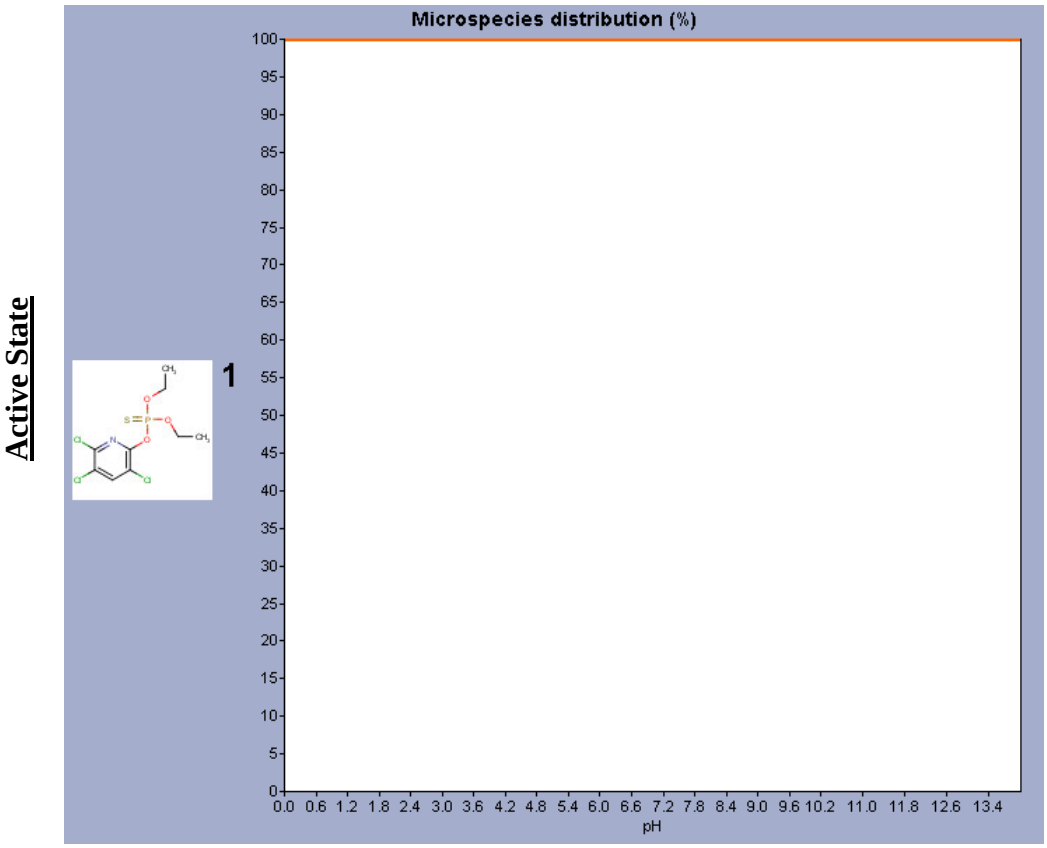
1. Bioallethrin pKa Determination– No ionizable atoms between pH1-14



Bioallethrin – logD Determination



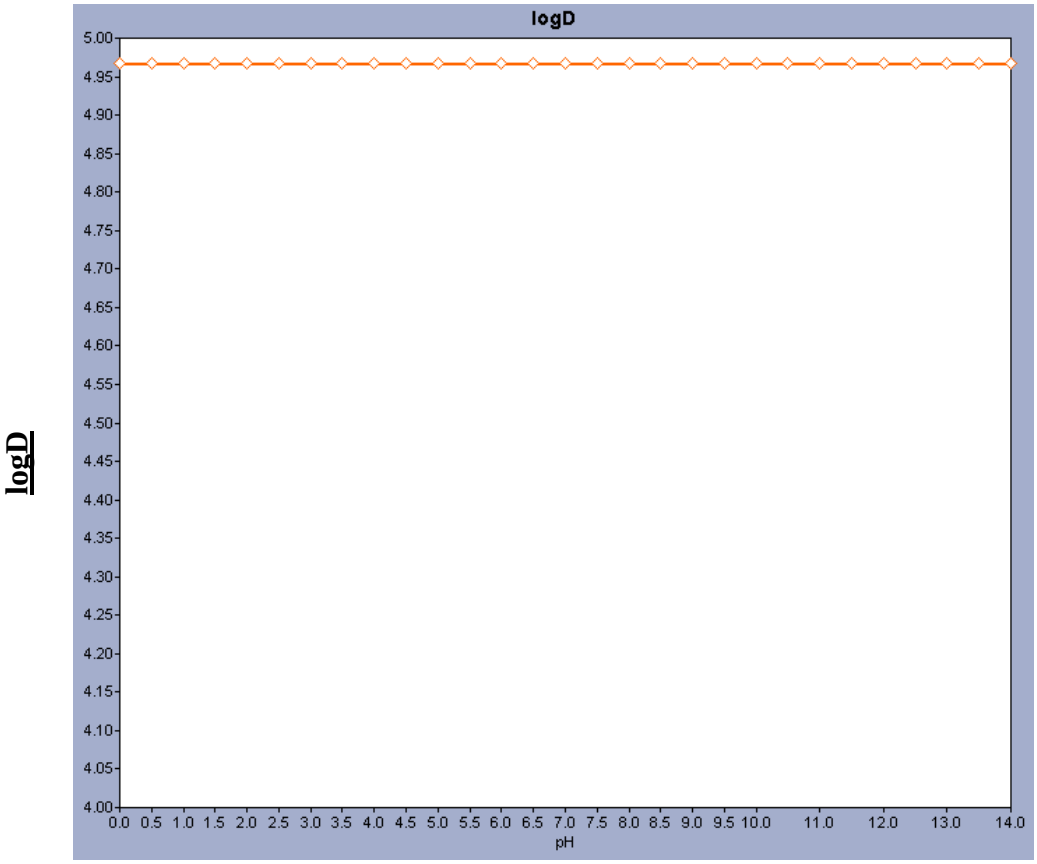
2. Chlorpyrifos pKa Determination – No ionizable atoms between pH1-14



Active State

pH

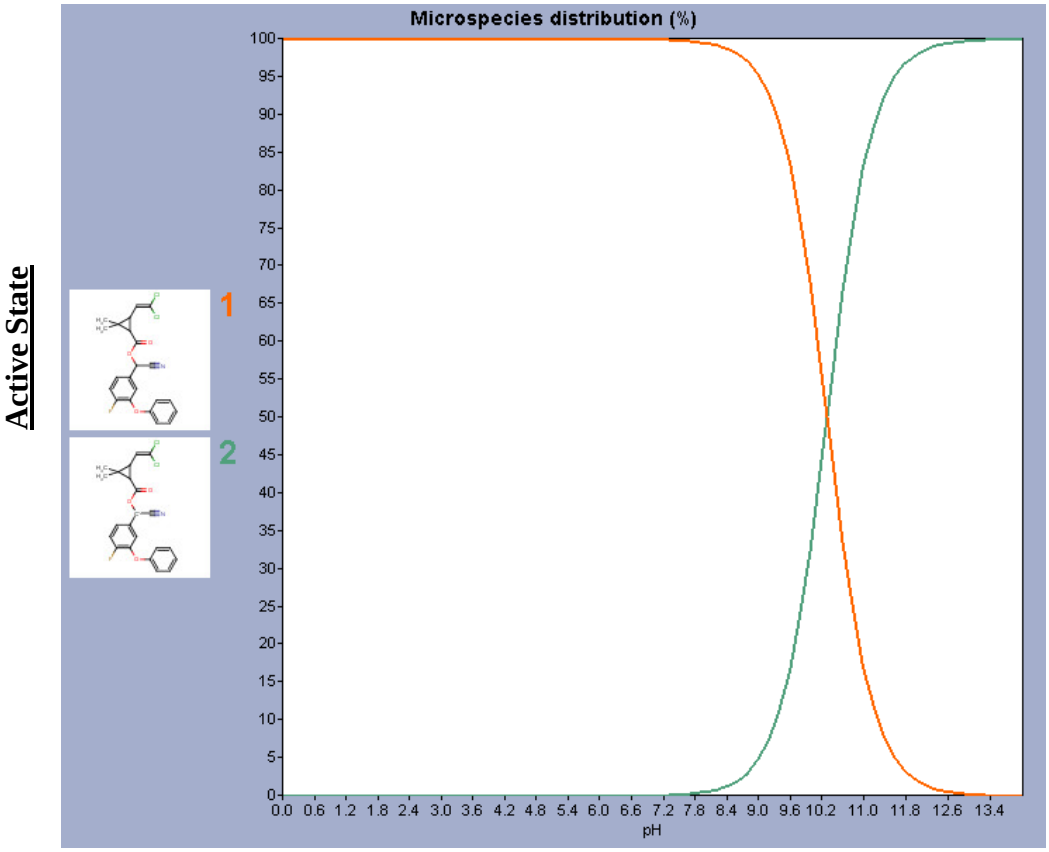
Chlorpyrifos – logD Determination



logD

pH

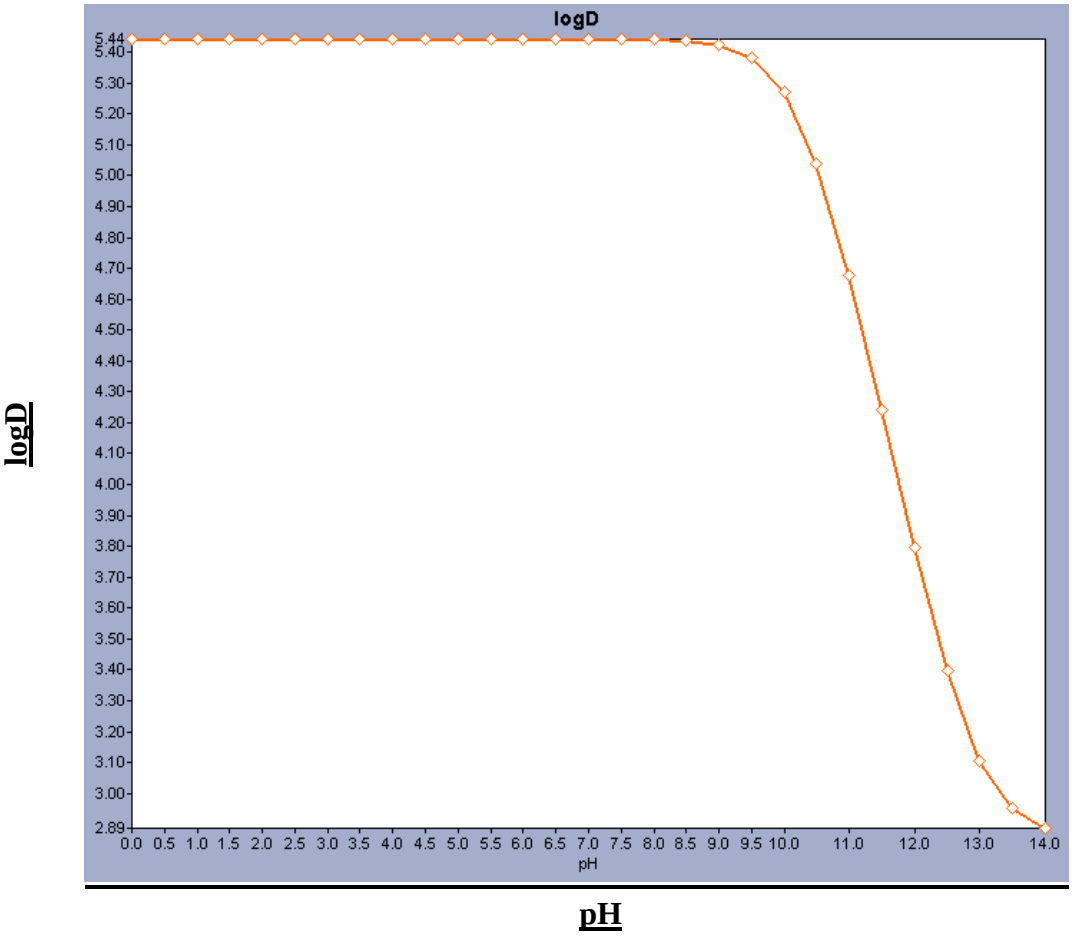
3. Cyfluthrin pKa Determination-1 ionizable atom between pH1-14



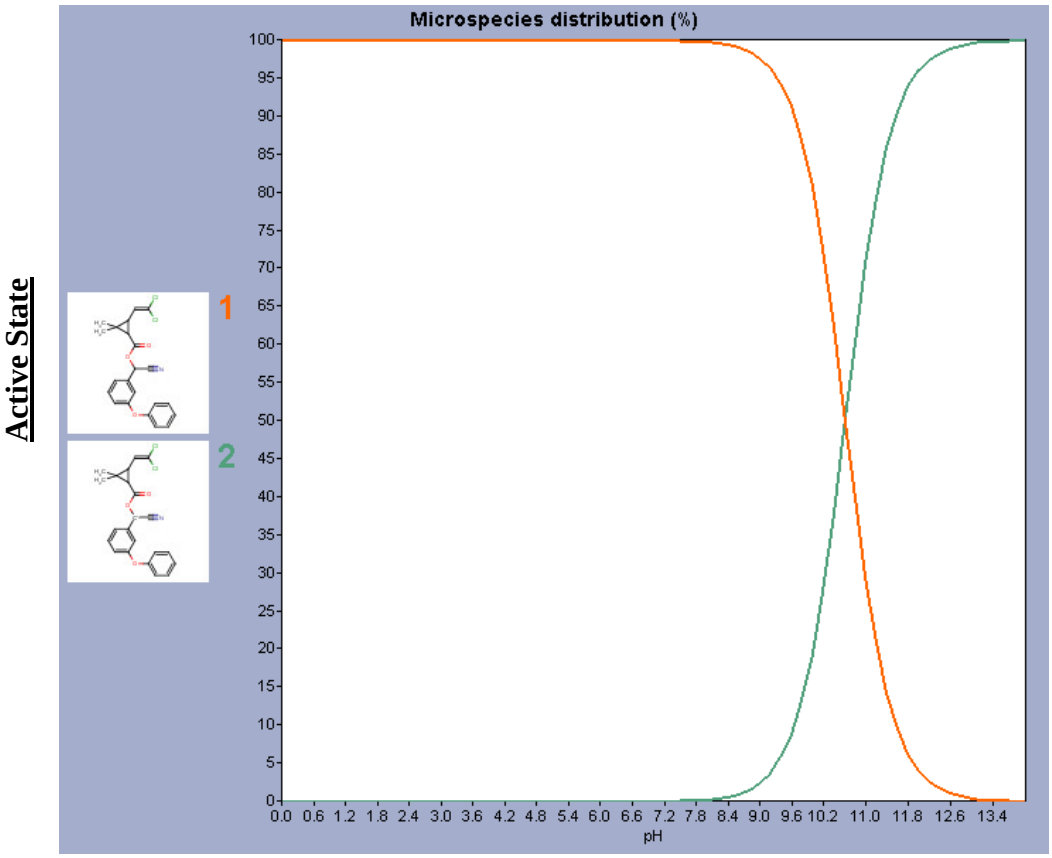
Active State

pH

Cyfluthrin – logD Determination



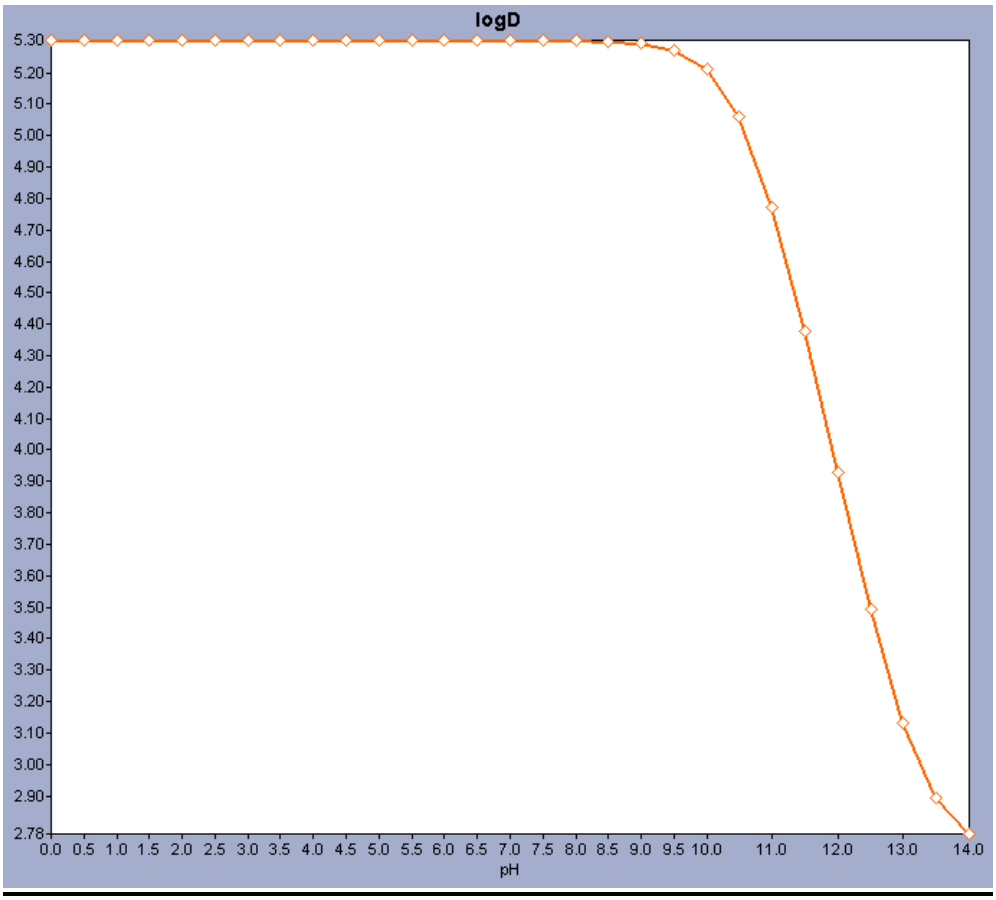
4. Cypermethrin pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

Cypermethrin – logD Determination

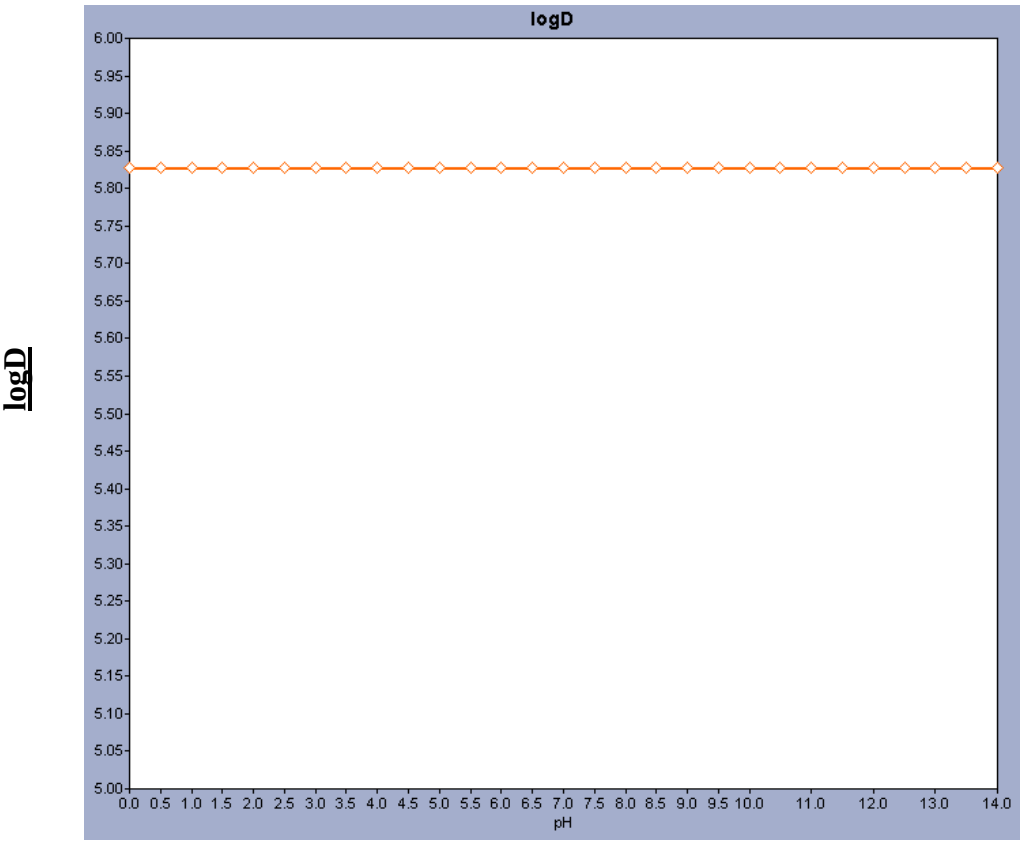


logD

pH

5. DDE pKa Determination – no ionizable atoms (no figure generated)

DDE - logD Determination

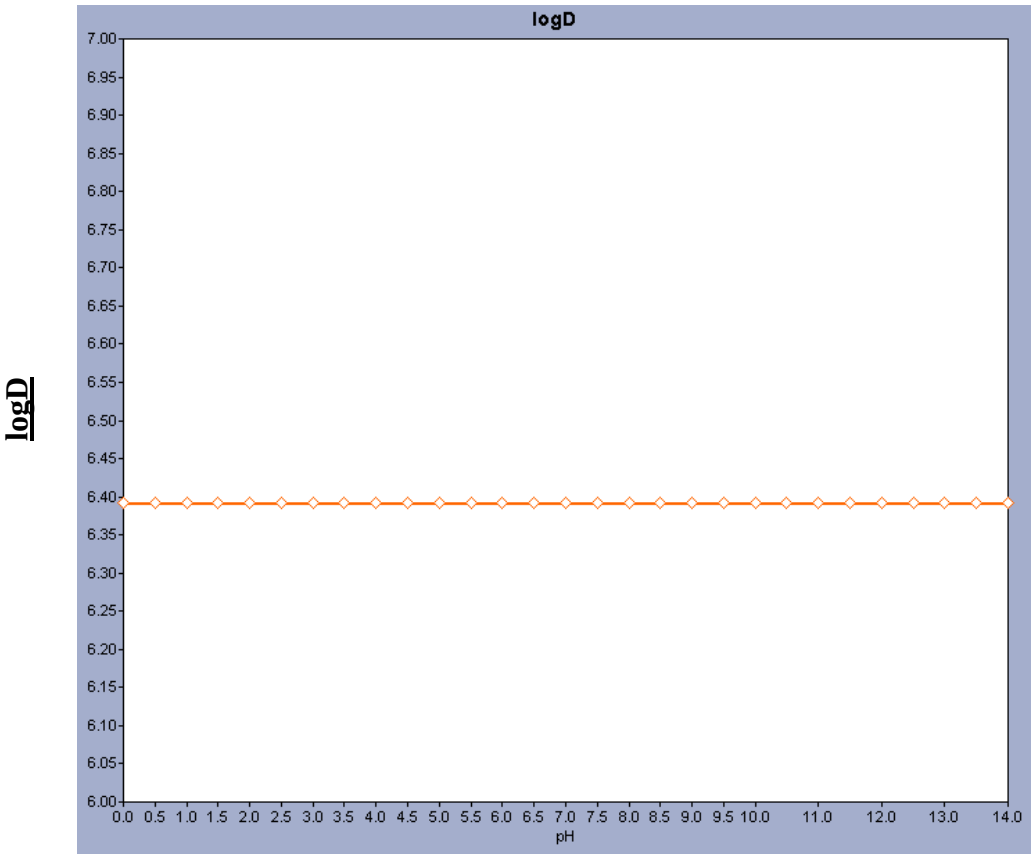


logD

pH

6. DDT pKa Determination – no ionizable atoms (no figure generated)

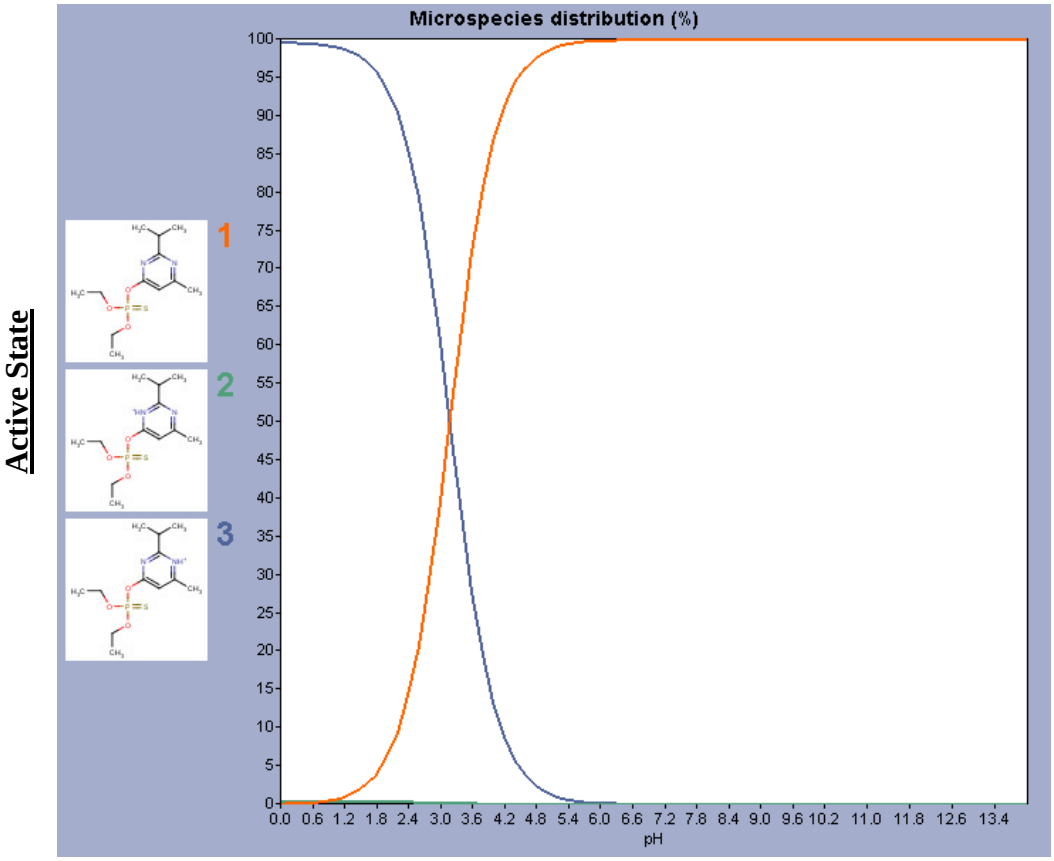
DDT - logD Determination



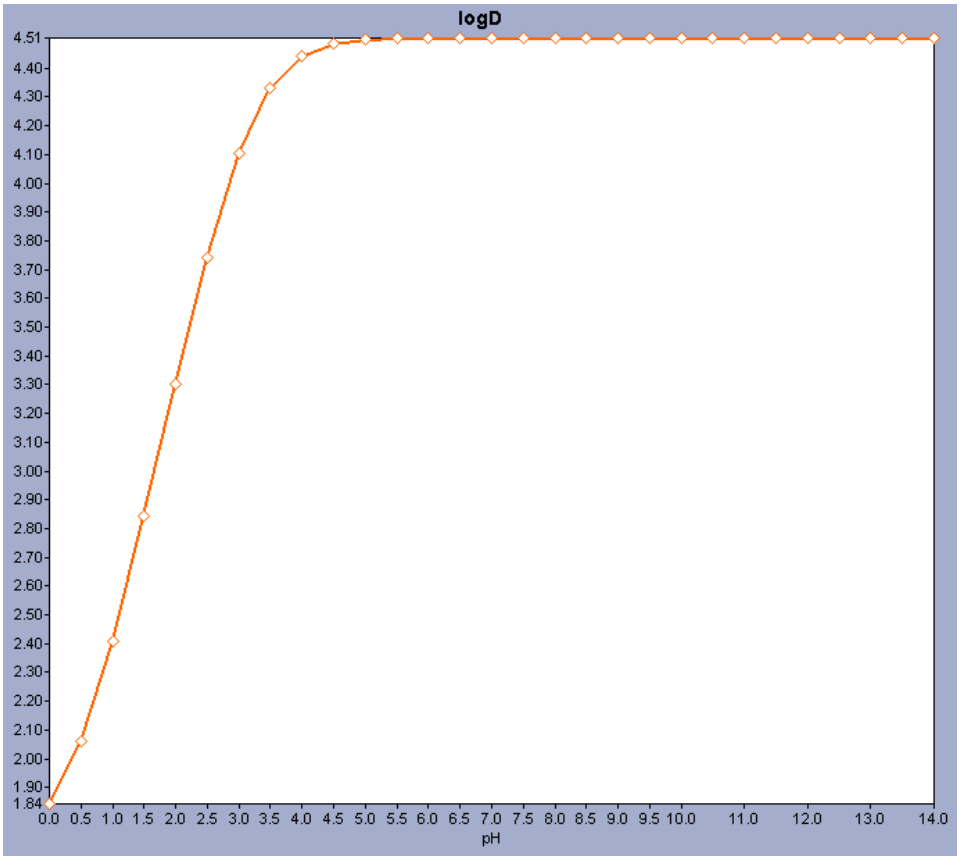
logD

pH

7. Diazinon pKa Determination – 1 ionizable atom between pH1-14



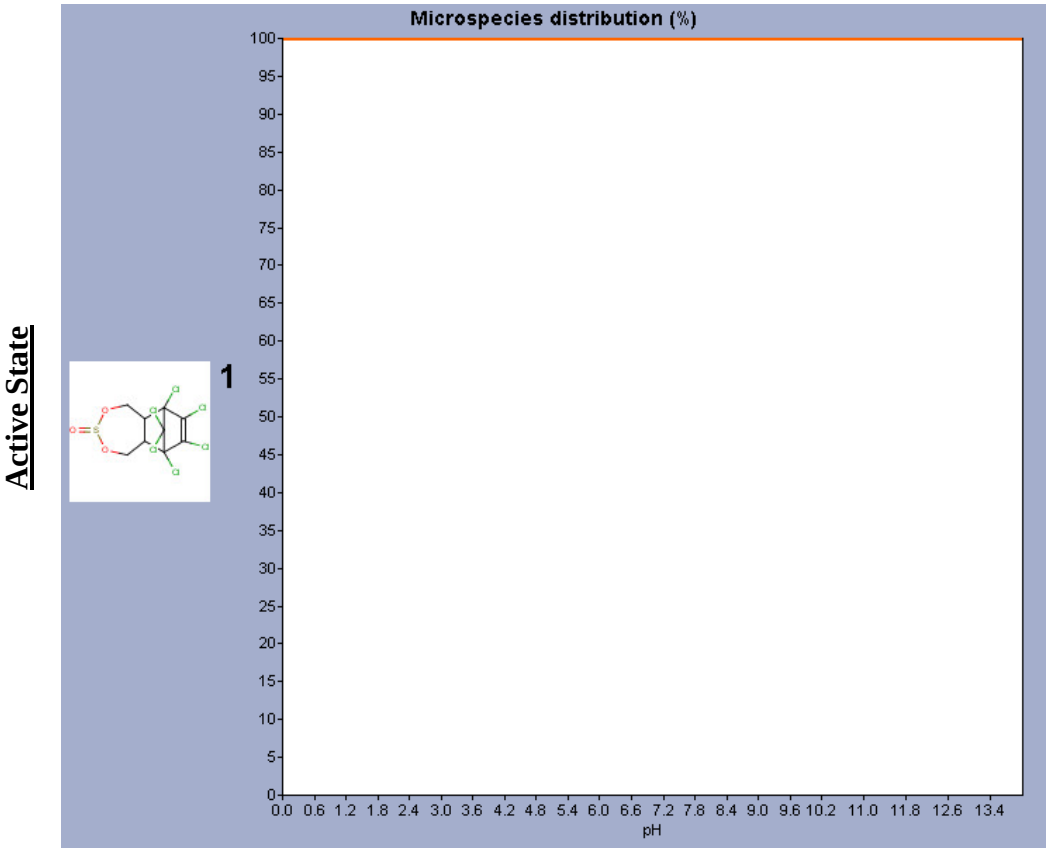
Diazinon - logD Determination



logD

pH

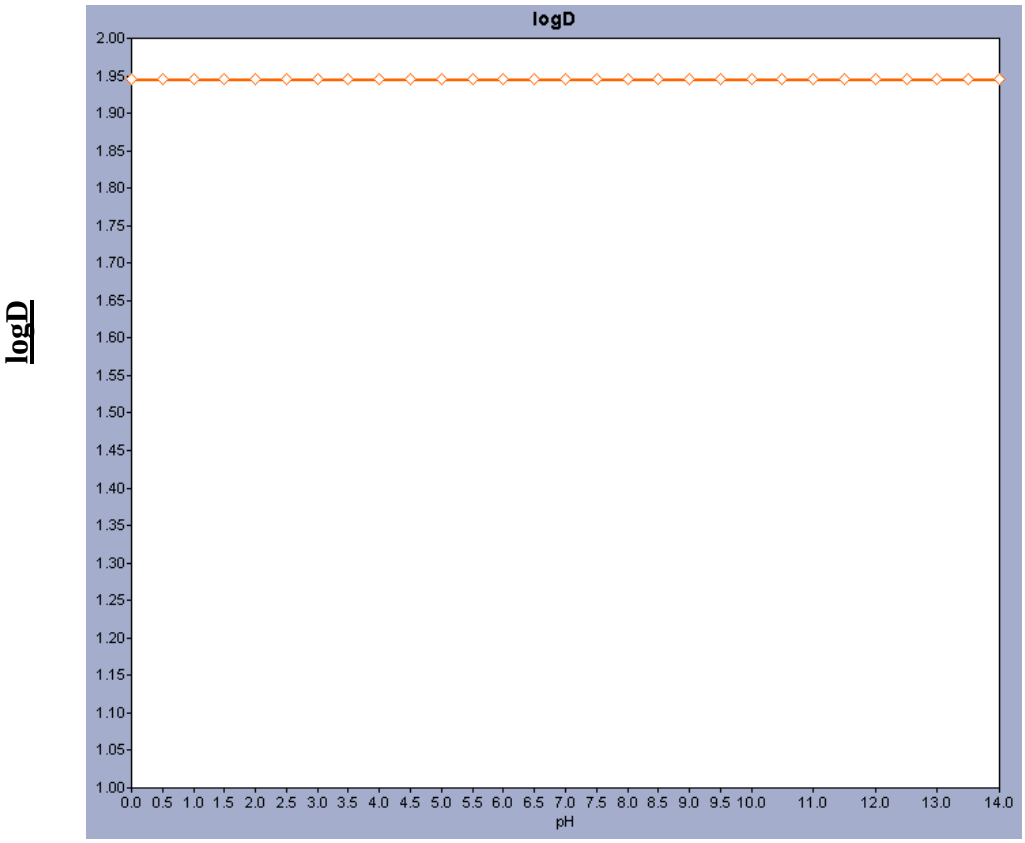
8. Endosulfan pKa Determination - no ionizable atoms between pH1-14



Active State

pH

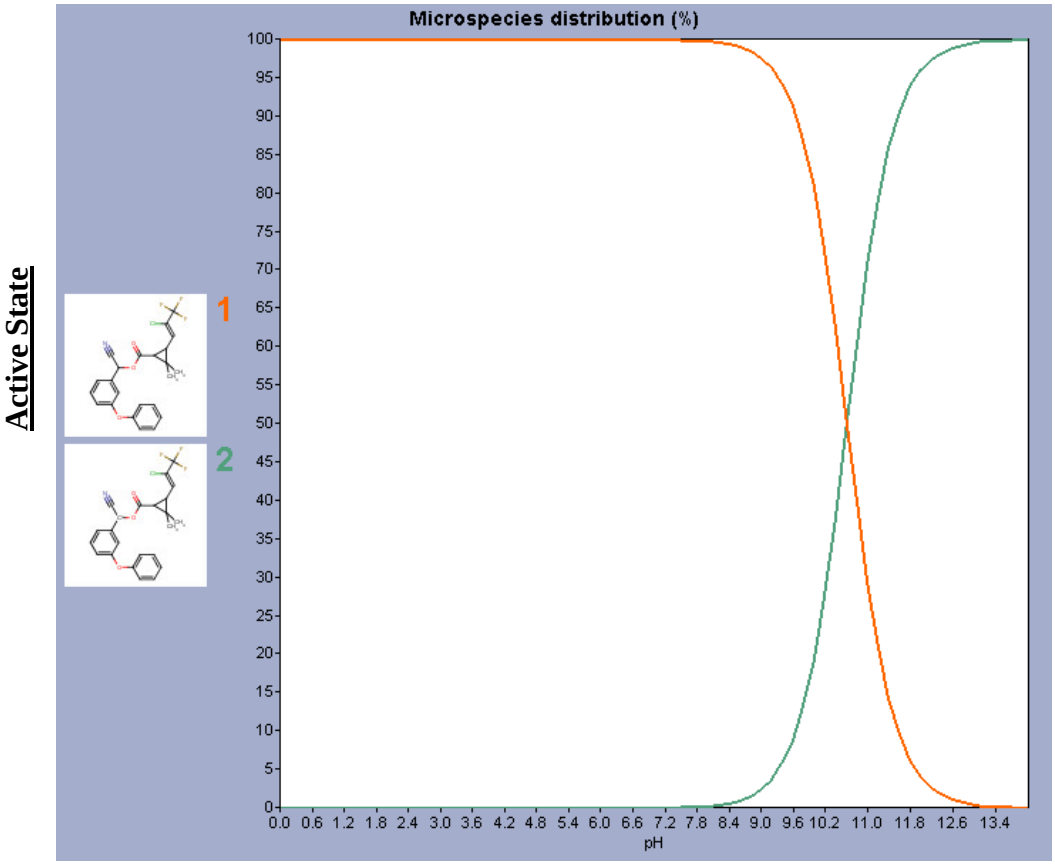
Endosulfan - logD Determination



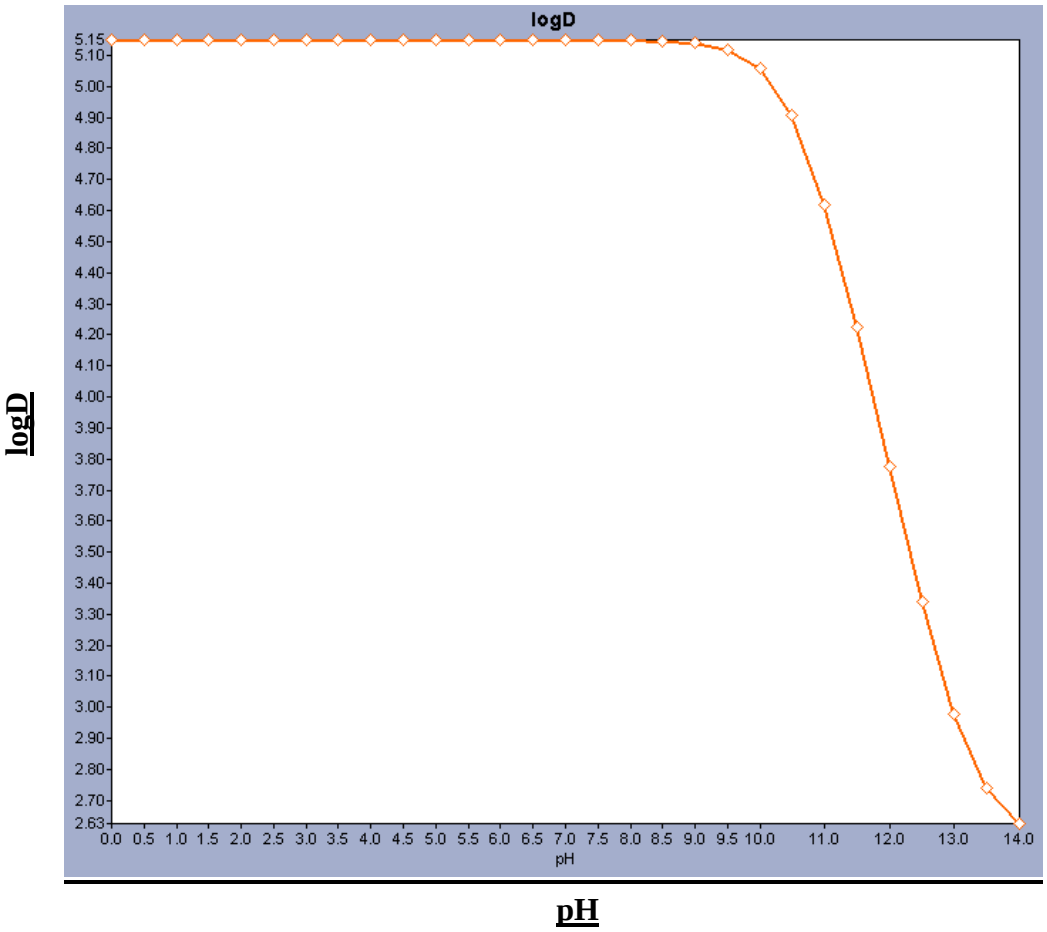
logD

pH

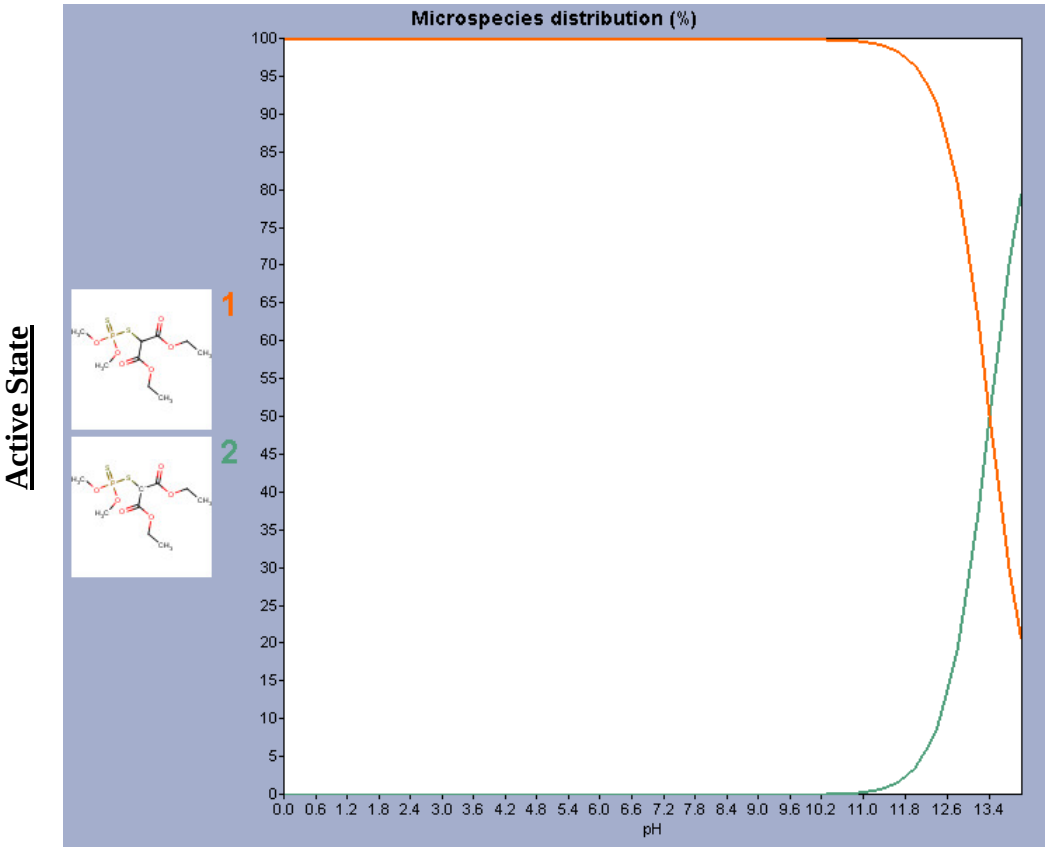
9. Lambda Cyhalothrin pKa Determination– 1 ionizable atom between pH1-14



Lambda Cyhalothrin - logD Determination



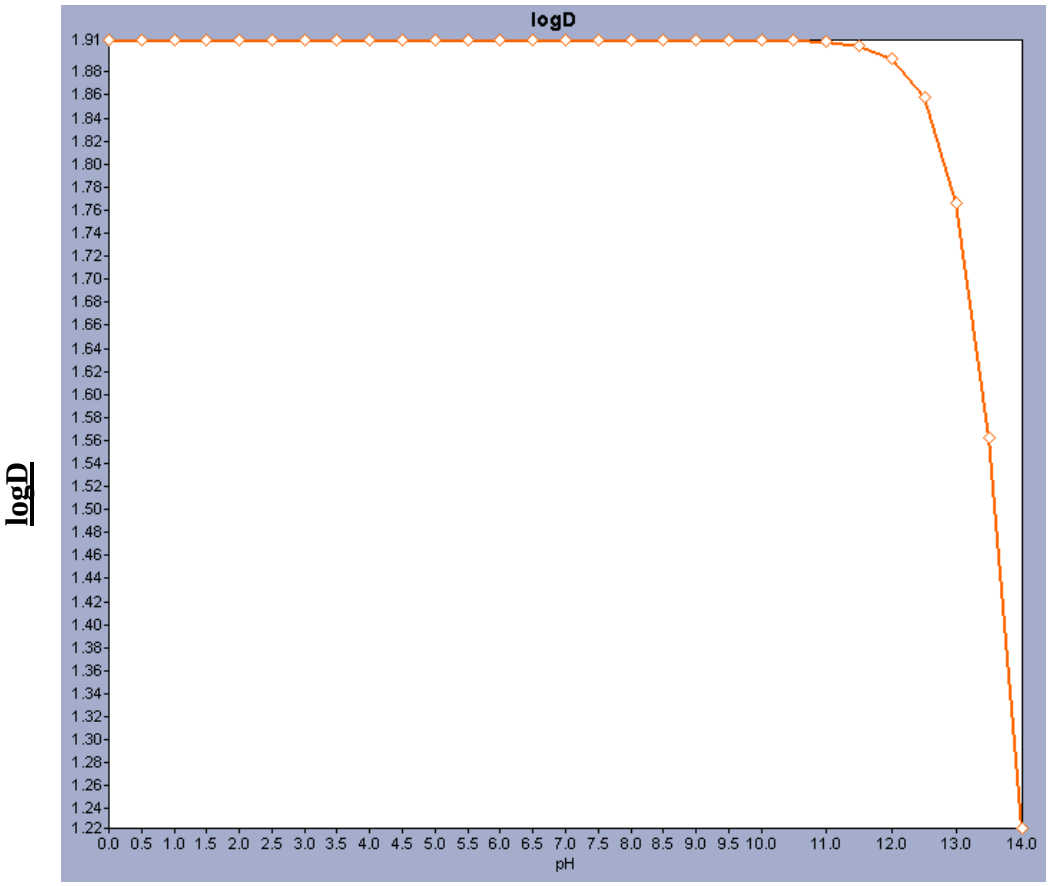
10. Malathion pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

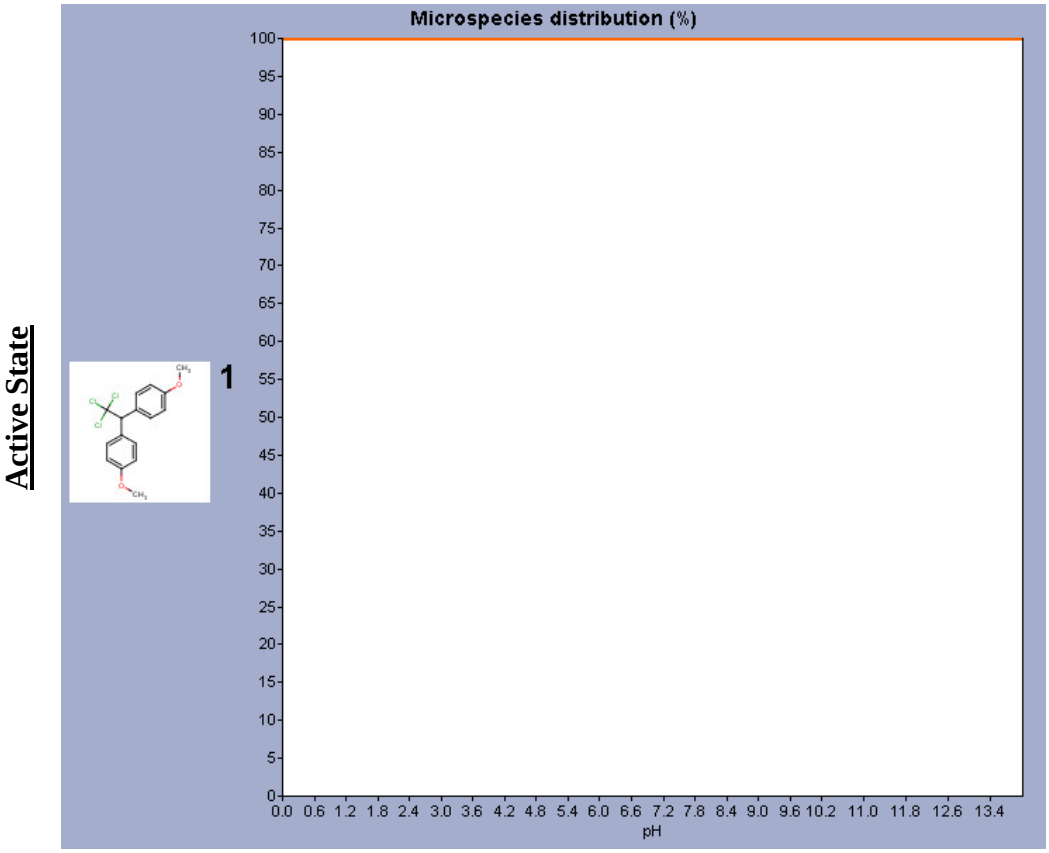
Malathion - logD Determination



logD

pH

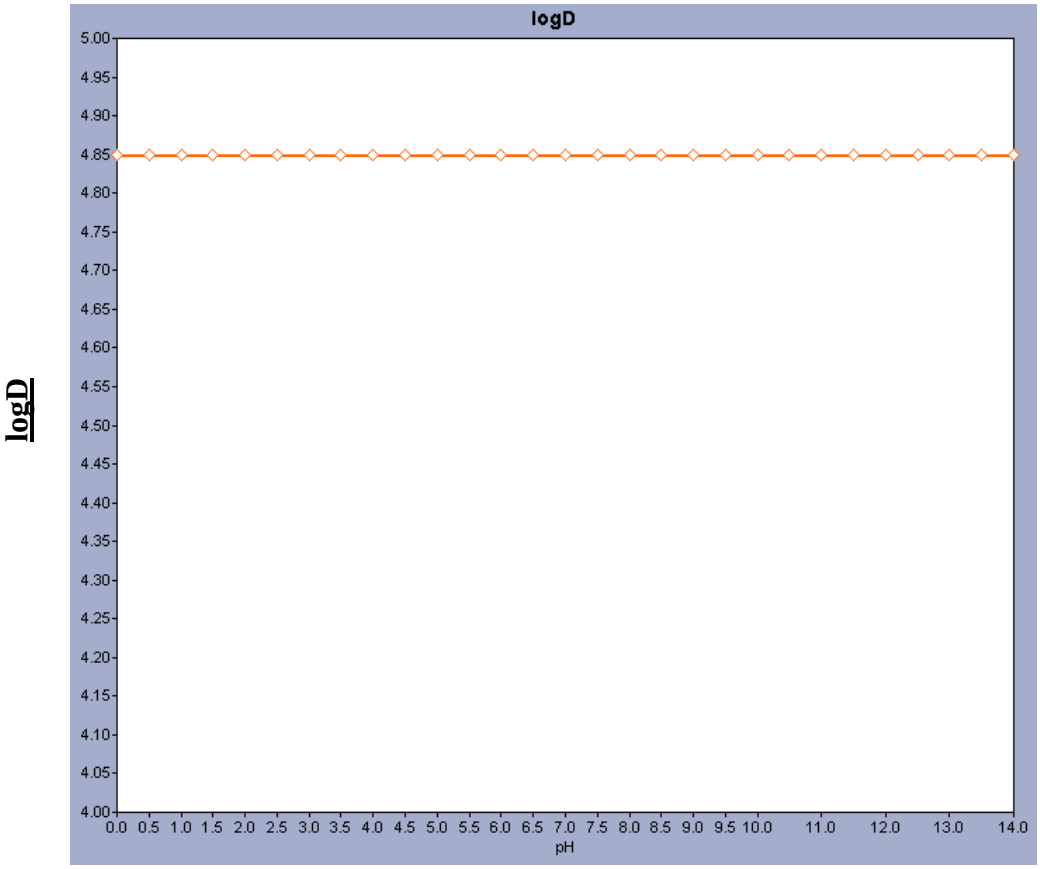
11. Methoxychlor pKa Determination – no ionizable atoms between pH1-14



Active State

pH

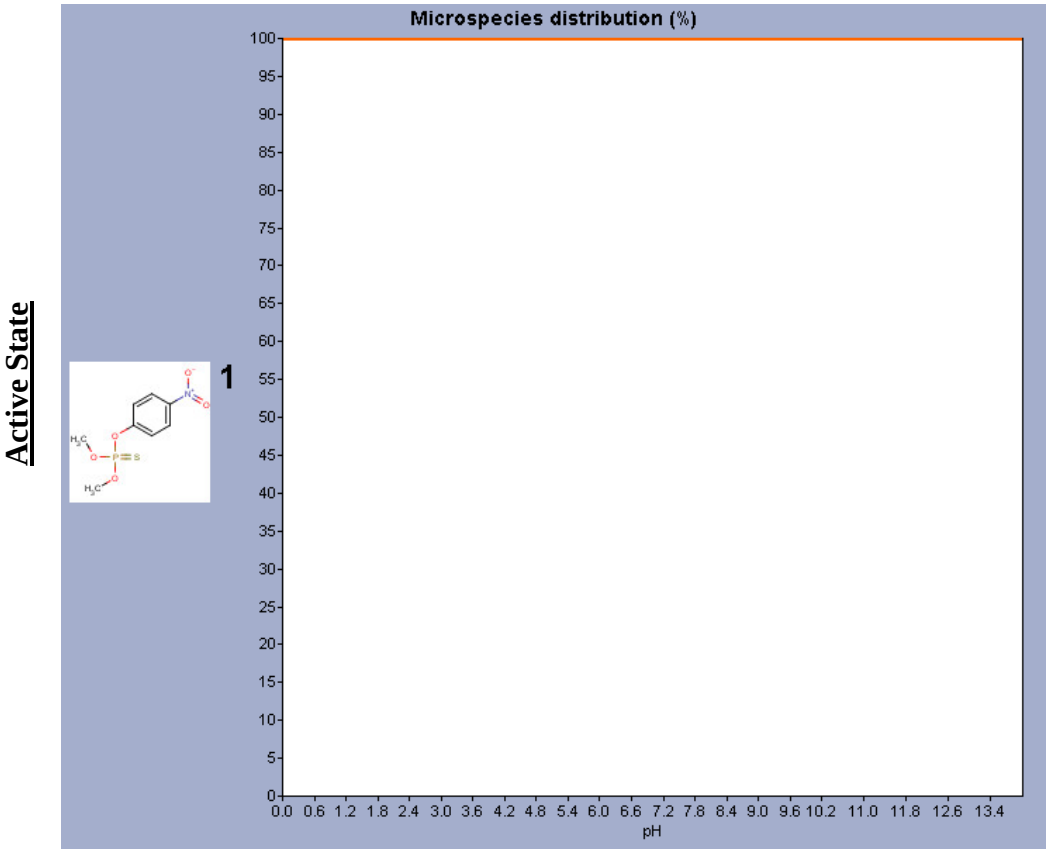
Methoxychlor - logD Determination



logD

pH

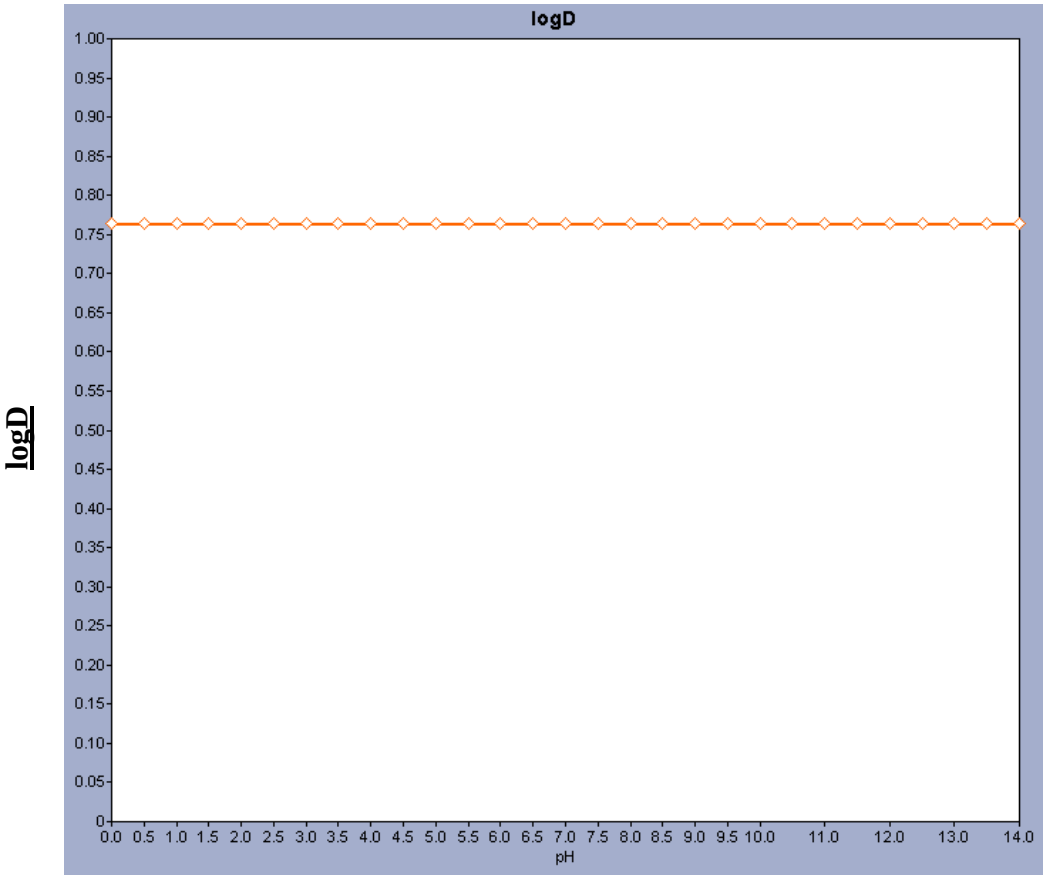
12. Methyl Parathion pKa Determination – No ionizable atoms between pH1-14



Active State

pH

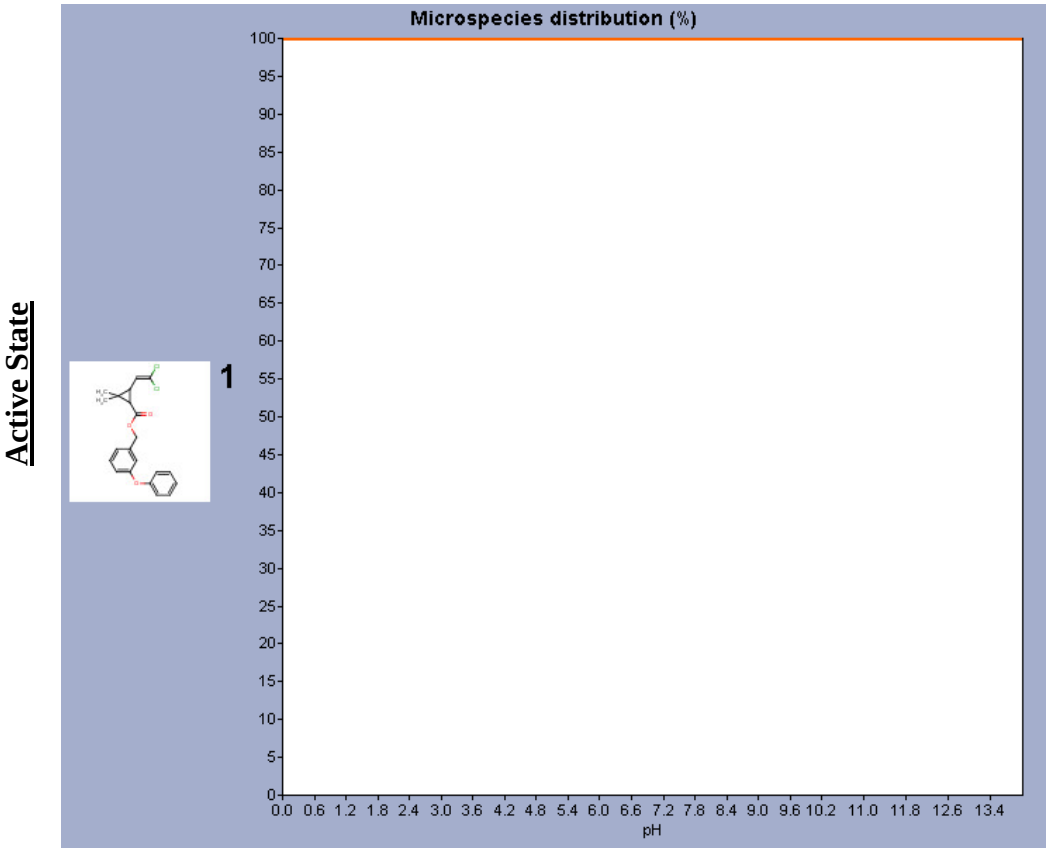
Methyl Parathion - logD Determination



logD

pH

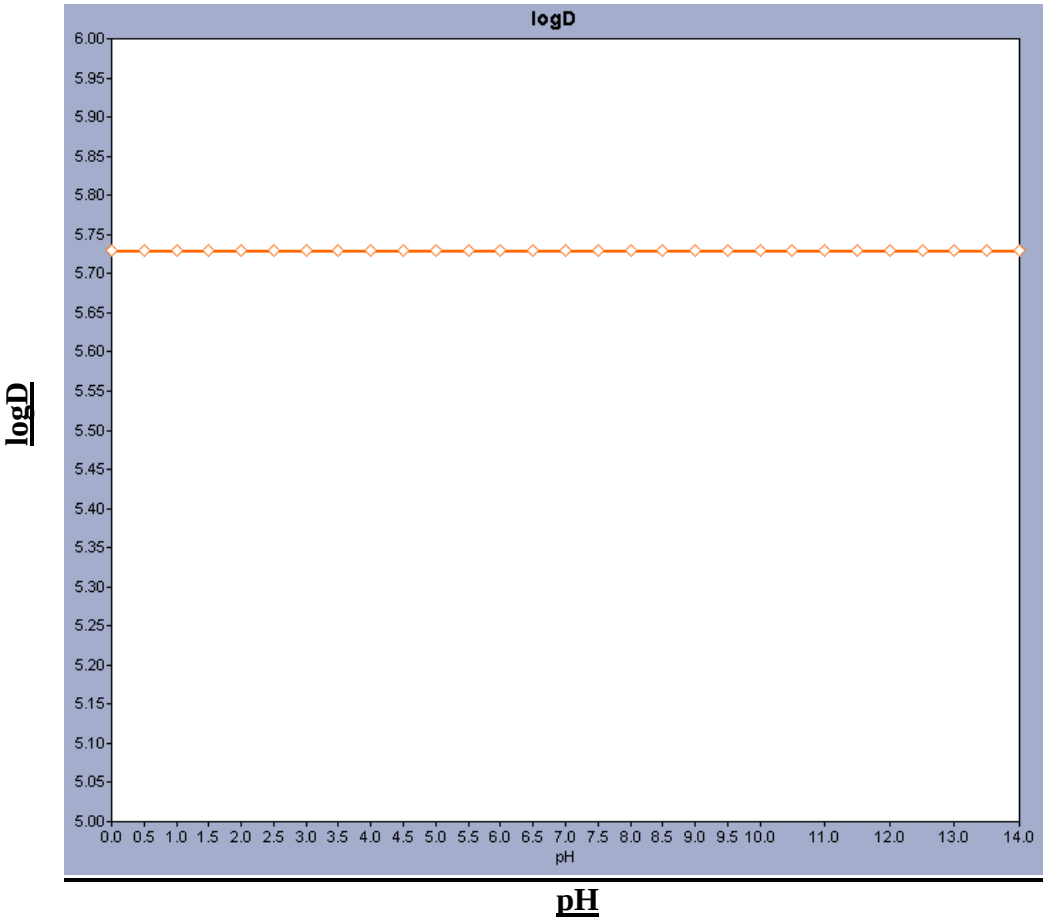
13. Permethrin pKa Determination – No ionizable atoms between pH1-14



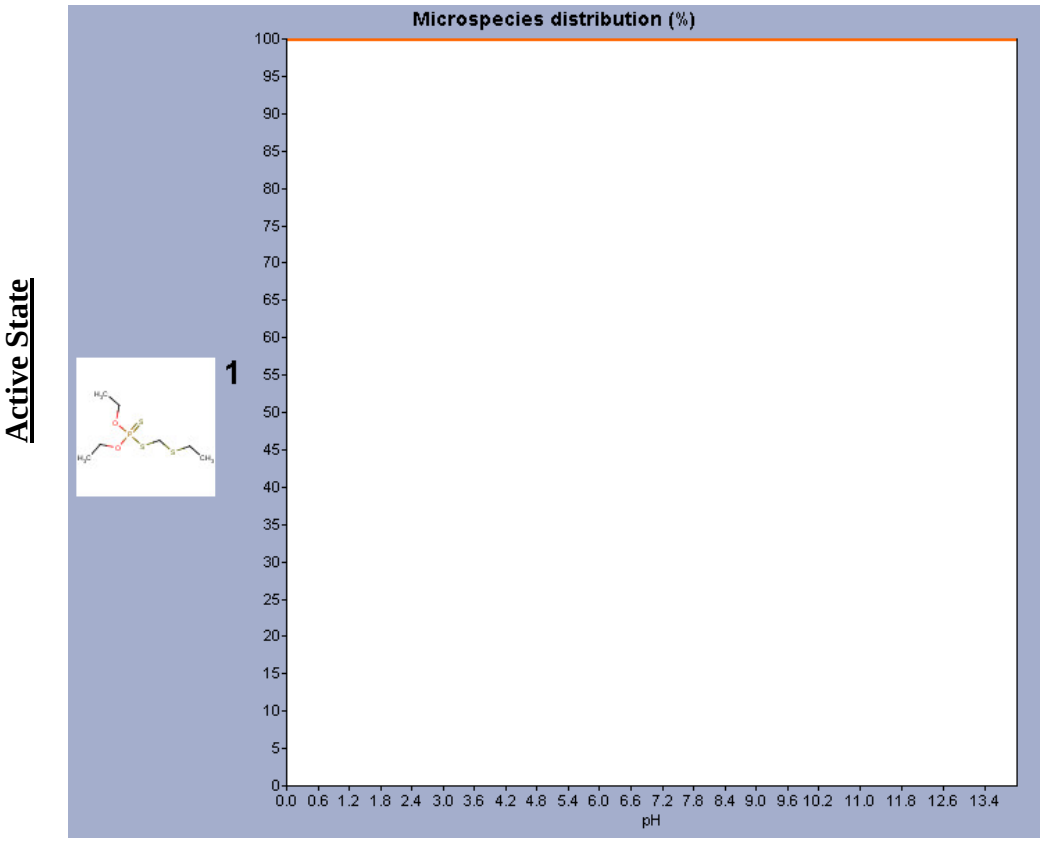
Active State

pH

Permethrin - logD Determination



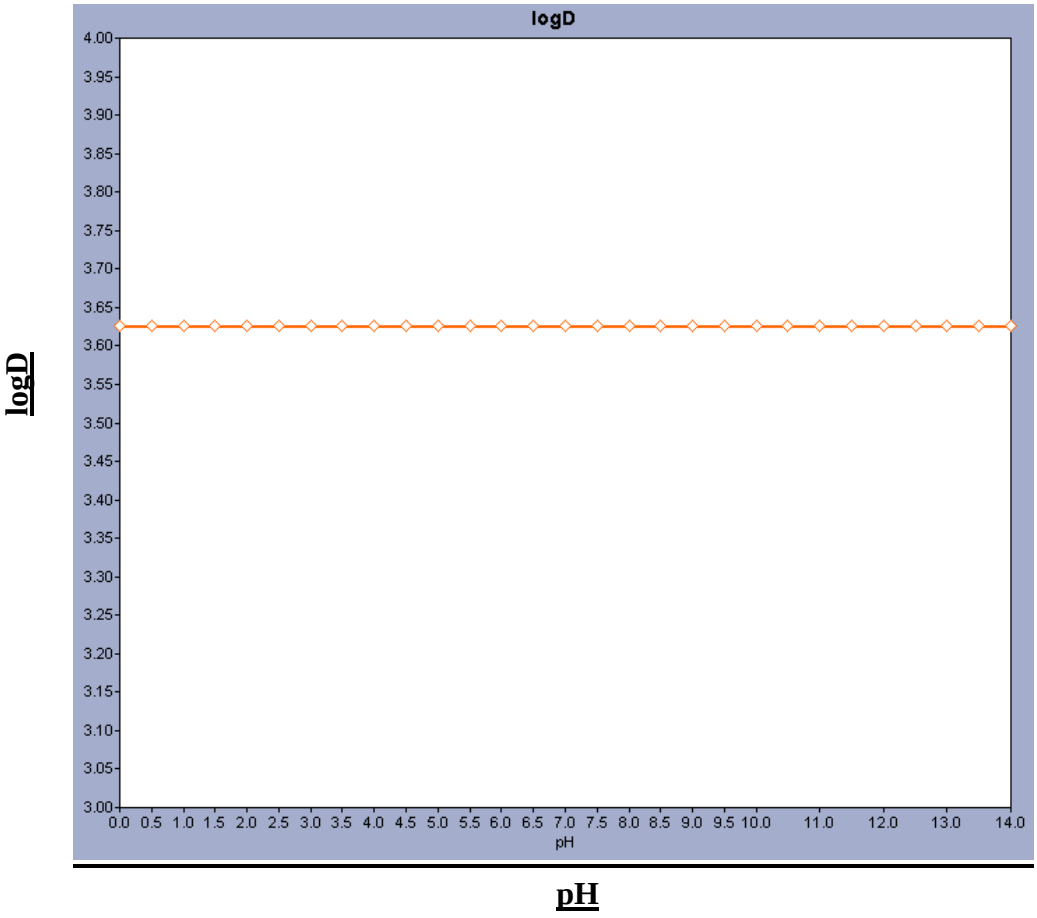
14. Phorate pKa Determination – No ionizable atoms between pH1-14



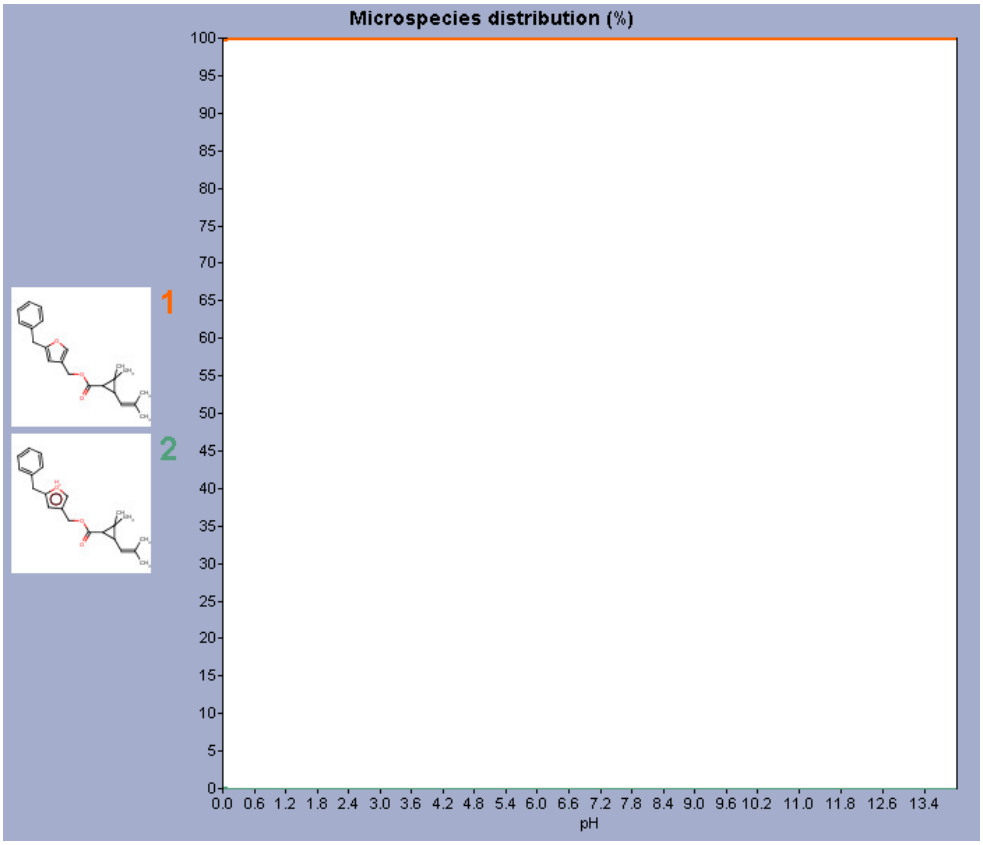
Active State

pH

Phorate - logD Determination



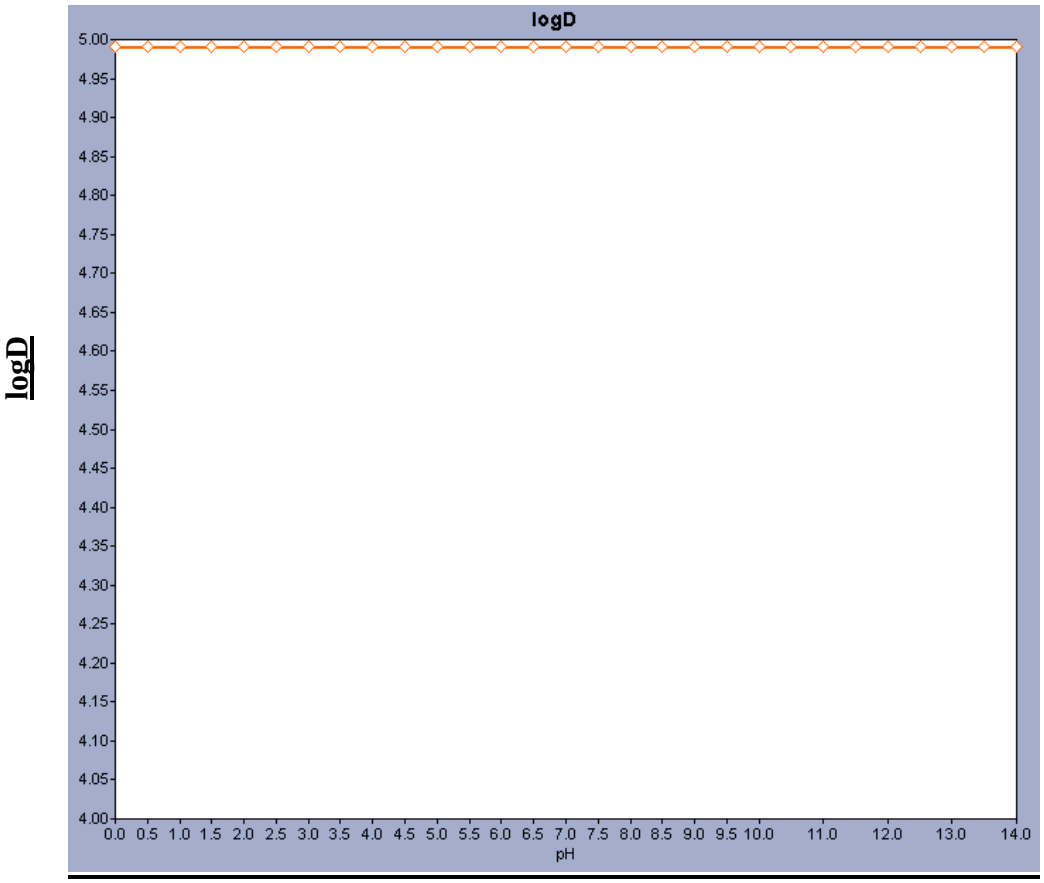
15. Resmethrin pKa Determination – No ionizable atoms between pH1-14



Active State

pH

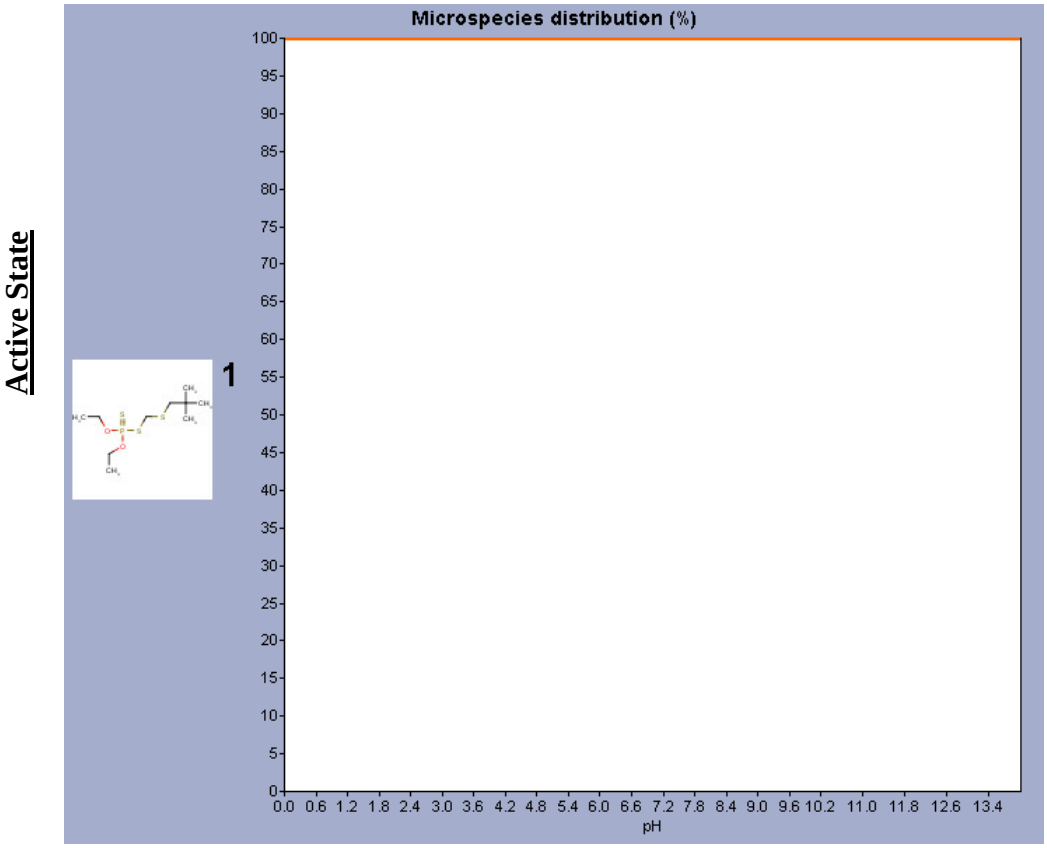
Resmethrin - logD Determination



logD

pH

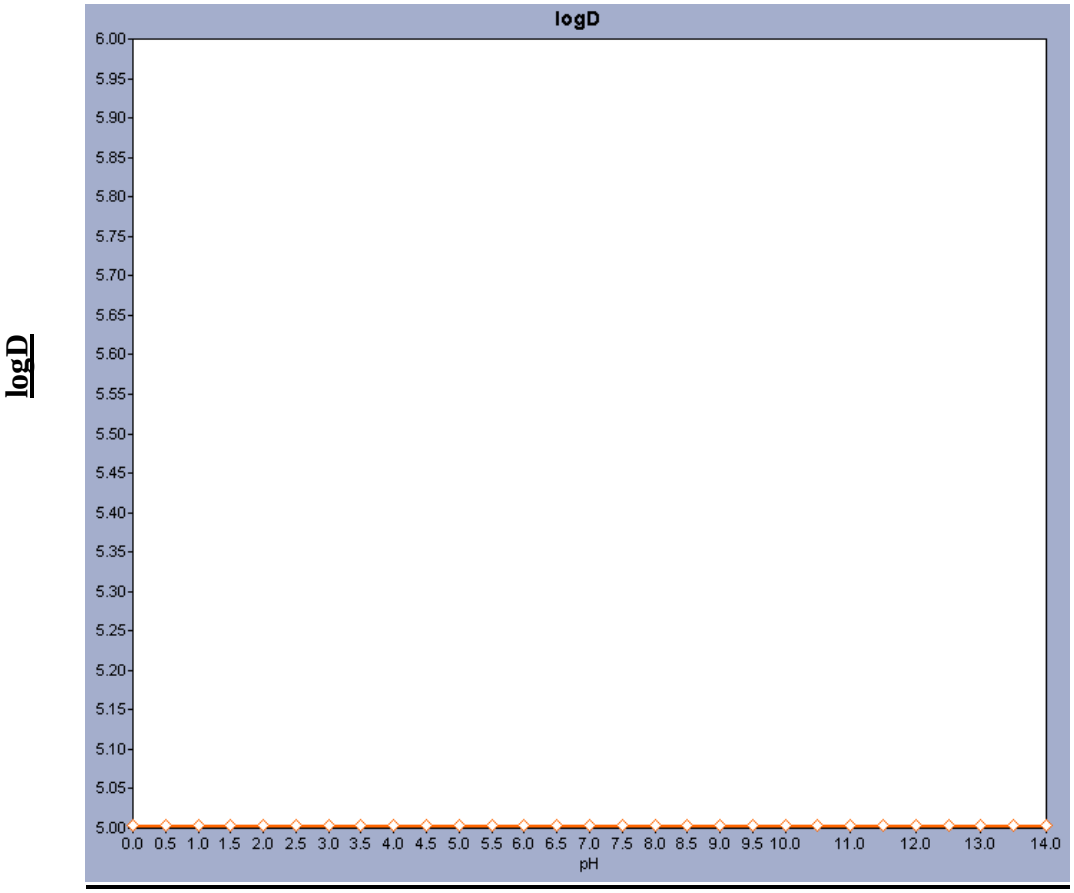
16. Terbufos pKa Determination – No ionizable atoms between pH1-14



Active State

pH

Terbufos - logD Determination



logD

pH

CHAPTER 3: ANALYSIS OF PESTICIDE DEGRADATION PRODUCTS IN SOIL

Introduction

The National Academy of Sciences 1993 report on Pesticides in the Diets of Infants and Children highlighted the specific vulnerabilities of children and emphasized the need for the development of new risk assessment methods that would provide insight and clarity into children's exposures to pesticides (NRC 1993; Landrigan 2004). Specifically, multi-chemical and multi-media pathways need to be evaluated, an intricate task that has never been accomplished. Because children differ from adults in their consumption rates (more intake than adults pound per kilogram of body weight), and immature metabolic and developmental pathways, they are considered to be at heightened risk from exposure than adults (Landrigan 2004). The United States Congress integrated the report's concepts into the Food Quality Protection Act (FQPA) of 1996, the major governing statute that regulates pesticide usage in agriculture (FQPA 1996). Expanding current exposure assessment methods to include exposures from multiple chemicals and from multiple routes is just one of the provisions of the Act to better determine exposure assessments of children to pesticides. This task is complex considering the multiple variables that need to be evaluated. For example, during the course of one day, a child spends varying amounts of time at different locations performing various activities. The isolation of a single exposure becomes difficult when attempting to pinpoint exact logistics of children and their behaviors. There is an additional challenge in determining exposure to transient, non-persistent pesticides like pyrethroids and organophosphates that have shorter half lives than other pesticides. The time of sample collection then becomes essential to understanding the exposure. Adding to the complexity is a more recent issue: the accuracy of the currently accepted and applied model for assessing pesticide exposure

(currently organophosphate pesticides). The model assumes that measuring the metabolites in the body are good markers of exposure to parent pesticides in the environment. However, the model is obscured by the fact that OP and pyrethroid breakdown products in the environment are usually the same chemical as the metabolite measured in the body. Potential contribution of degradation products to the metabolite concentration found in people needs to be assessed. Understanding the fate of these pesticides and their degradation products individually in the environment is the first step in determining what their effects on people and the environment will be. Additionally, the degradation products of these pesticides have unknown toxicity and longevity in the environment and additional studies are needed to answer these questions. Understanding pesticide degradation product behavior in various environmental media from dust, soil, water to food products is an important concept that needs to be integrated into overall exposure assessments. The result will be a more comprehensible understanding of multiple-chemical exposure to children, a key requirement to the FQPA.

In order to understand the fate of these pesticides, analytical methods need to be developed to accurately measure and quantitate these chemicals as markers for exposure. There are various analytical techniques to measure pesticides in their parent form in multiple environmental media. However, the analysis of pesticide degradation products in environmental media for the purpose of exposure assessment is a novel concept. For this research, soil will be the matrix under investigation for degradation product analysis because it is an accessible matrix in both agricultural and residential settings and is a typical matrix exposed to pesticide sprayings. The first step to analyze degradation

products in soil is to determine which degradation products need to be analyzed. There are several environmental processes (hydrolysis, photolysis, microbial degradation, oxidation, and reduction) and factors (soil makeup, pH and weathering) that influence how a pesticide will transform and transport through environmental matrices, making it difficult to determine which products to investigate. Nonetheless, there are numerous studies that have determined through modeling or *in vitro* analysis what major degradation products result from these processes in soil (Laveglia 1977; Howard 1991; Roberts 1999; Bavcon, Trebse et al. 2003). A list of degradation products included for this research was determined from a collation of commonly-applied residential and agricultural pesticides used today and their known degradation products and is shown in Table 3.1. The structures for these degradation products are shown below in Figures 3.1-3.3.

			Soil Degradation Pathway				
Parent Pesticide	Class	Major Soil Degradation Products[a]	Microbial	Hydrolysis	Oxidation	Photolysis	Metabolic Product[b]
Methyl Parathion	OP	4-Nitrophenol	Yes	Yes			Yes
Diazinon	OP	1-Isopropyl-6-methly-4pyrimidol (IMPY)		Yes		Yes	Yes
Malathion	OP	Malathion dicarboxylic acid (MDA)	Yes	Yes			Yes
Chlorpyrifos	OP	3,5,6 Trichloro-2-pyridinol (TCPY)	Yes	Yes		Yes	Yes
Phorate	OP	Sulfoxide	Yes		Yes		No[d]
Phorate	OP	Sulfone			Yes		No[d]
Terbufos	OP	Sulfoxide			Yes		No[d]
Terbufos	OP	Sulfone			Yes		No[d]
Multiple Organophosphates	OP	Dimethylphosphate (DMP)	Yes	Yes			Yes
	OP	Diethylphosphate (DEP)[c]		Yes			Yes
	OP	Dimethylthiophosphate (DMTP)	Yes	Yes			Yes
	OP	Dimethyldithiophosphate (DMDTP)	Yes	Yes			Yes
	OP	Diethylthiophosphate (DETP)		Yes			Yes
	OP	Diethyltdithiophosphate (DEDTP)		Yes			Yes
Cyfluthrin	Pyrethroid	4-Fluoro-3-phenoxybenzoic acid (4F3PBA)		Yes		Yes	Yes
Cypermethrin, Permethrin,Cyfluthrin	Pyrethroid	3-(2,2-Dichlorovinyl)-2,2- dimethylcyclopropane carboxylic acid (DCCA)		Yes		Yes	Yes
10/18 available pyrethroids in US	Pyrethroid	3-Phenoxybenzoic acid (3 PBA)		Yes		Yes	Yes
Deltamethrin	Pyrethroid	*cis* -3-(2,2 dibromovinyl)-2,2 dimethylcyclopropane-1-carboxylic acid (DBCA)				Yes	Yes
Resmethrin	Pyrethroid	*cis/trans* Chrysanthemum carboxylic acid (CDCA)		Yes			Yes
Cyhalothrin, Bifenthrin	Pyrethroid	3,3,3 Trifluoroprop-1-enyl-2,2 dimethylcyclopropane carboxylic acid (CFCA)		Yes			Yes

[a] Minor degradation products not included in study

[b] As detected in urinary analysis

[c] Found as a degradation product of plant hydrolysis of several organophosphates

[d] Found in minute quantities in urine, however considered as toxic if not more toxic and more persistent than individual parent compounds.

Table 3.1 Degradation Pathways and Products of Parent Pesticides. (Laveglia 1977; Racke and Coats 1988; Howard 1991; Kaur, Mathur et al. 1997; Leng, Kuhn et al. 1997; Roberts 1999; Hong, Win et al. 2001; Bravo, Driskell et al. 2002; Bavcon, Trebse et al. 2003; Olsson, Nguyen et al. 2003; Singh, Singh et al. 2003; Chuang, Van Emon et al. 2004; Labana, Pandey et al. 2005)

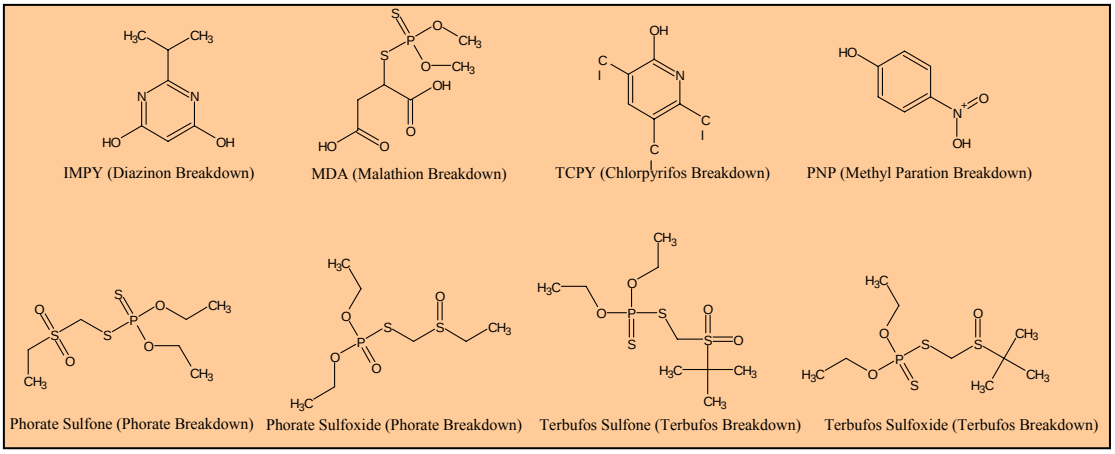


Figure 3.1 Structures of Specific OP Degradation Products.

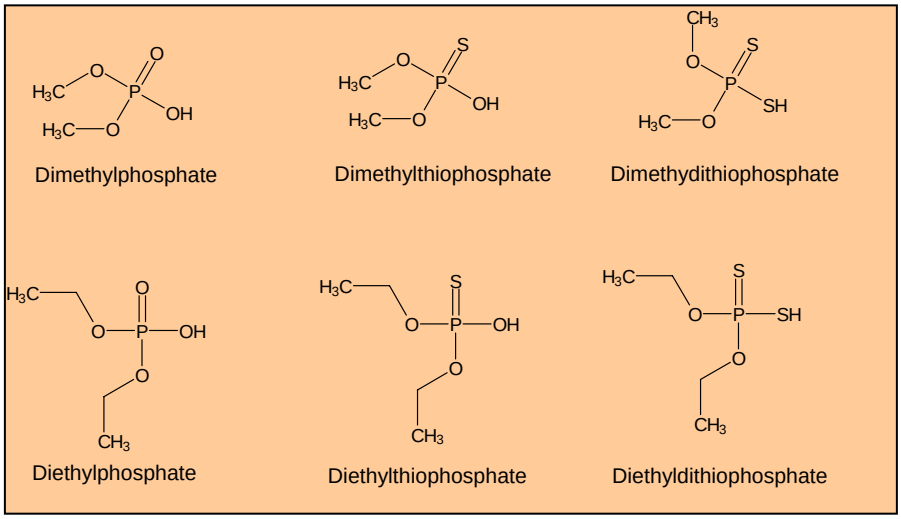


Figure 3.2 Structures of Non-Specific OP Degradation Products.

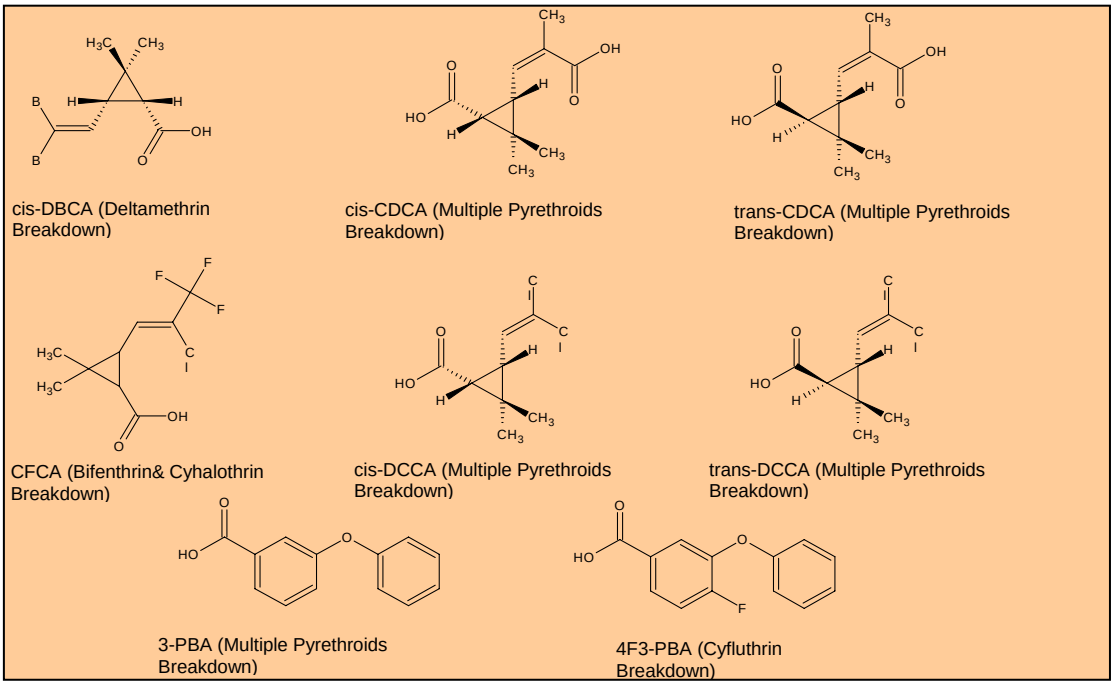


Figure 3.3 Structures of Pyrethroid Degradation Products.

Development of a reliable analytical method for degradation products in soil is important to the public for two reasons: exposure model assumptions, and toxicity and persistence of degradation products.

Exposure Model Assumptions

The Committee on Human Biomonitoring for Environmental Toxicants, an independent study group formed by the National Research Council (NRC), prepared a research agenda due to increasing concerns governing US public health policies in response to biomonitoring data and studies (NRC 2006). In evaluating current biomonitoring studies and their impact on public health, the committee concluded that, "Epidemiologic, toxicologic, and exposure assessment studies have not adequately incorporated biomonitoring data for interpretation of health risks at the individual, community and population levels" (NRC 2006). Specifically, what are the implications of exposure data to the public health? How does the public interpret biomonitoring studies? If environmental toxicant metabolites are present in significant amounts in a cohort study, how should these measurements be interpreted? Modern methods of exposure rely heavily on biological monitoring which reveal body burden concentrations (Barr, Wang et al. 2005; Bradman and Whyatt 2005). These methods have been based on the assumption that pesticide metabolites result from direct exposure to the parent compounds through either environmental or dietary means. However, environmental degradation also occurs, releasing various breakdown products into different environmental matrices. In many if not most cases, the degradation product is the same chemical as the metabolic breakdown being used as a biomarker for parent pesticide

exposure. Consideration of breakdown products as possible contributors to the presence of metabolic breakdown product found in humans needs to be taken into consideration when correlating pesticide exposure and metabolite presence as biomarkers of exposure. If environmental degradation products lead to higher apparent concentrations of metabolites in people, then the current method leading to the direct correlation between parent pesticide exposure and metabolite formation is confounded by the possibility of being exposed to the degradation products directly themselves. This is an area of exposure assessment that needs to be investigated and clearly defined before making assumptions between exposure and health effect.

Several studies display evidence of this potential misclassification. Morgan *et al*. found that 2-isopropyl-6-methyl-4-pyrimidinol (IMPY), a urinary metabolite of diazinon, and 3,5,6 trichloro-2-pyridinol (TCPY), a urinary metabolite of chlorpyrifos, was found in several environmental media (soil, dust, air, hand wipes, food) (Morgan 2004; Morgan, Sheldon et al. 2005). TCPY was found in concentrations ten times higher than chlorpyrifos concentrations in solid food samples (Morgan 2004). In addition to these findings, they determined that levels of the parent products did not correlate with their corresponding biomarkers indicating that these metabolites were generated from another source. They concluded that biomarker analysis for measuring exposure to organophosphates be re-evaluated. MacIntosh *et al.* found only 7% of TCPY in urine samples from their study population that could be attributed to dietary intake of chlorpyrifos (Macintosh, Kabiru et al. 2001). The group concluded that dietary exposure to chlorpyrifos was not a major contributor to urinary TCPY concentrations and other

sources of TCPY may contribute to overall urinary metabolite concentrations. Zhang *et al.* also found that diakylphosphates (DAPs-non-specific breakdown products of organophosphates) in fruits and vegetables had much larger concentrations than their parent pesticide counterparts (Zhang, Barr et al. 2005). In fact, they determined that 91 out of 153 samples contained more dialkylphosphates than their parent organophosphates (Zhang, Barr et al. 2005). They concluded that current biomarker analysis substantially overestimates the amount of parent OP exposure. Lu *et al*. found levels of preformed dialkylphosphates in both conventional and organic orange and apple juices (Lu, Bravo et al. 2005). Lastly, in an Environmental Health Perspective rebuttal of a study published by Curl *et al*. that measured DAPs in the urine of children in an attempt to differentiate between pure organic and conventional diets, Robert Krieger challenged the study findings claiming that the study results overestimated DAPs found in the urine of conventional dieters and thus exploited the benefits of organic diets (Curl, Fenske et al. 2003; Krieger, Dinoff et al. 2003). Krieger argued that the group directly associated high DAP results with being exposed to parent pesticide without taking into consideration that children are probably being exposed to nontoxic DAPs in the diet in addition to the metabolic product of the parent pesticide found in the diet contributing to overall DAP concentration found in the urine. In another study by Lu *et al.,* a dramatic reduction in urinary DAPs concentrations resulted from children who switched to organic diets for 5 consecutive days (Lu, Toepel et al. 2006). This publication conveyed to the public less risk associated with consuming organic diets. However, the higher presence of DAPs in children while they consumed conventional dietary sources did not delineate if the DAPs came from exposure to the parent OP pesticides or the OP degradates (DAPs) themselves.

The obscure distinction led to alternating rebuttals between scientists from various groups regarding what scientific terminology should be used to interpret results so as to not mislead the public (Avery 2006; Krieger, Keenan et al. 2006; Lu, Fenske et al. 2006; Lu, Toepel et al. 2006). Differentiating between exposure to preformed DAPs in environmental media versus exposure to parent pesticide and subsequent metabolism to DAPs needs to be clarified before making the assumption that higher urinary DAP levels automatically mean higher exposure to OP pesticides.

In conclusion, the existing exposure model needs to be re-evaluated taking into account possible contributions of environmental degradation products to biomarker concentrations used to determine pesticide exposure. As a result, biomonitoring exposure studies will more accurately define exposure and also help clarify the pathways of pesticide degradation/metabolism. Although pyrethroid biomarker analysis has not been as intensely evaluated as the case with OP exposure, urinary biomarkers that estimate pyrethroid exposure are the same compounds found as degradates in environmental studies and should be included in any exposure model considerations. The latest US National Health and Nutrition Examination Survey (NHANES) report issued in 2005, which estimates the general population to pesticides and other environmental toxicants of concern, suggests that more than 50% of the US population is exposed to pyrethroid insecticides due to the presence of 3-PBA, a common urinary metabolite from exposure to common residential pyrethroids, permethrin, cypermethrin and deltamethrin (Table 3.2) (CDC/NCEH 2005). If this widespread exposure is due to direct exposure to the parent insecticides or the degradate itself has yet to be determined.

	Survey Years	Geometric Mean (95% Confidence Interval)	Selected Percentiles (95% Confidence Interval)				
			50th	75th	90th	95th	Sample Size
Total, age 6 and older	2001-2002	0.321 (0.276-0.374)	0.280 (0.220-0.340)	0.690 (0.560-0.810)	1.690 (1.41-2.33)	3.32 (2.52-5.25)	2539
AGE GROUP							
6-11 years	2001-2002	0.325 (0.260-0.406)	0.300 (0.200-0.410)	0.750 (0.560-1.03)	1.81 (1.34-2.69)	3.28 (2.25-4.12)	580
12-19 years	2001-2002	0.353 (0.288-0.434)	0.290 (0.250-0.390)	0.800 (0.560-1.13)	1.85 (1.48-2.35)	3.45 (2.14-6.69)	831
20-59 years	2001-2002	0.314 (0.271-0.364)	0.270 (0.210-0.340)	0.670 (0.530-0.780)	1.64 (1.27-2.34)	3.25 (2.51-6.16)	1128
GENDER							
Males	2001-2002	0.328 (0.277-0.387)	0.290 (0.230-0.370)	0.680 (0.560-0.750)	1.55 (1.26-2.16)	3.23 (2.56-5.78)	1193
Females	2001-2002	0.315 (0.266-0.373)	0.250 (0.210-0.320)	0.730 (0.530-0.920)	1.76 (1.47-2.35)	3.28 (2.34-6.16)	1346
RACE/ETHNICITY							
Mexican Americans	2001-2002	0.297 (0.238-0.369)	0.250 (0.190-0.340)	0.650 (0.480-0.810)	1.30 (0.830-2.26)	2.71 (1.51-3.44)	680
Non-Hispanic Blacks	2001-2002	0.507 (0.428-0.601)	0.510 (0.430-0.630)	0.950 (0.840-1.12)	2.00 (1.65-2.28)	3.25 (2.52-4.62)	701
Non-Hispanic Whites	2001-2002	0.298 (0.246-0.362)	0.230 (0.180-0.320)	0.590 (0.470-0.800)	1.72 (1.27-2.46)	3.38 (2.25-7.64)	957

Table 3.2 NHANES 2005 Report Summary of 3-PBA Exposure *Adapted from (CDC/NCEH 2005).*

Toxic and/or Persistent Degradation Products

Although dialkylphosphates are considered nontoxic (however, evidence is lacking to refute or support this), there may be metabolites or degradation products that have been found to be as toxic and persistent if not more so than their parent pesticide counterparts. For example, phorate, an agricultural toxic insecticide, is an organophosphate whose degradation products, phorate sulfoxide and phorate sulfone are much more persistent and at least as toxic than phorate itself (Racke and Coats 1988; Kamrin 1997; Hong, Win et al. 2001). Although this may be beneficial for insecticidal activity, it may prove to be problematic when considering human exposure. Phorate has been shown to rapidly oxidize anywhere from 1 hour to several days whereas the degradation products, the sulfone and sulfoxide persist from 1-4 months (Kamrin 1997). It is also evident that acetylcholinesterase activity increases from the parent pesticide phorate to the sulfoxide to the most persistent, sulfone (Kamrin 1997). 4-nitrophenol is a degradation product of methyl parathion that is listed by the United States Environmental Protection Agency as a priority pollutant because of its high toxicity (Labana, Pandey et al. 2005). Tyler *et al.* stressed the need to understand environmental degradation of pesticides (pyrethroids for

this study) in order to assess toxicities, specifically endocrine-modulating activities, when they found 3-phenoxybenzoic acid (degradation product of multiple pyrethroids) to have anti-estrogenic activity (Tyler, Beresford et al. 2000). In a recent publication, three degradation products/metabolites from cypermethrin and permethrin, two commonly used household insecticides, were found to be weakly estrogenic in a recombinant yeast assay measuring 17β-estradiol activity further strengthening the argument that the toxic effects of pesticide degradation products need to be further evaluated and understood (McCarthy, Thomson et al. 2006).

There has been scarce attention paid to toxic degradates when determining exposures to pesticides. If toxic degradates/metabolites were found in higher amounts in the body of conventional dieters versus organic dieters, the argument made by Krieger *et.al.,* would prove irrelevant because, although in theory nontoxic dialkylphosphates would not physically affect conventional dieters, toxic degradates might have an altogether different toxicological side effect. Degradation products should be identified as separate chemicals with different chemical and physical characteristics than their parent pesticide counterparts. Their behavior in the environment and in people needs to be assessed separately from the parent pesticides. In conclusion, the need for more epidemiological and toxicological research to answer these uncertainties is acutely evident.

Attention is being focused toward the analysis of degradation products in environmental and dietary matrices and a clear argument is being made. It is not only necessary to understand how exposure to parent pesticides in the environment and dietary means

affects people but it is also important to begin understanding how exposures to degradation products affect public health. Surveillance programs and national biomonitoring studies need to include degradation product exposure assessments in addition to parent pesticide assessments to understand comprehensively pesticide exposure outcomes. The first step in this debate is to develop accurate and robust methods to measure environmental degradate exposures. Analytical methods are the key that aid epidemiologists in their determination to find causal relationships between exposures and adverse health outcomes. Therefore, the primary focus of this section of the dissertation will be the development of an analytical method for analyzing degradation products in soil. In the future, novel methods will be developed to analyze degradation products through several media including food, which is likely to be the exposure route of greatest interest in humans. Existing methods that analyze degradation products only focus on one or two pesticides in relation to their fate and transport through soil. There have been to date no methods analyzing for a collective group of pesticide degradation products from multiple classes. As part of the proposed work, multiple organophosphate and pyrethroid degradation products will be analyzed in soil.

Method Development

Understanding the physical properties of the chosen degradation products is the first step to determine the best method for extraction from soil. Many studies examining these chemicals have been used for biological monitoring and epidemiological purposes looking at the chemicals as metabolites from exposure to the parent compounds. Therefore, methods detailing metabolite extractions from biological matrices will serve

as a starting point to understand their physical parameters. However, there are important differences to consider. Analysis of biological media, for example urine, is considerably different than soil. The major consideration when working with urinary matrices is the high and variable salt concentration which can hinder ion exchange mechanisms by which to extract ionizable compounds. Generally, urine is a simpler matrix with which to work; one can directly use solid phase or liquid-liquid extraction to extract target analytes, both of which are quick and effective extraction systems. Soil is a more difficult matrix to work with as it is a solid matrix which limits extraction methods. Soil extractions typically involve a form of solid-liquid extraction with subsequent clean-up procedures for the liquid extract. Ion exchange extractions may also be problematic with soil extracts as multiple salts exist in soils. Refluxing the soil with an organic solvent is typically the route chosen to extract chemicals from the soil such as microwave-assisted, sonication or Soxhlet extraction. Supercritical fluid extraction and accelerated solvent extraction are more novel techniques that convert soil into an easier to manipulate liquid matrix. Most of the degradation products chosen for examination are moderately-polar to polar analytes with respect to their parent compounds because their dominant degradation pathways, hydrolysis and photolysis, typically yield carboxylic acids and phenolic compounds (Howard 1991; Roberts 1999). In addition to higher polarity, these products are ionizable which increases options for investigating different extraction methods. For this research, investigation into conventional soil-shake extractions with polar solvents and subsequent clean-up of the degradation products will be performed narrowing in on differences between different post-extract clean-ups.

Another important factor to consider when developing analytical methods is how samples will be analyzed after extraction. Polar chemicals tend to be less volatile and therefore more difficult to separate using gas chromatographic techniques unless a derivatizing agent is used to enhance volatility. Derivitization can lead to problems because they are usually limited to one pesticide class and would be difficult with a combination of chemicals from multiple pesticide classes. As a result, liquid chromatography is the favored choice for analysis for this group of compounds. Additionally, insensitivity to thermal degradation, a necessary parameter for gas chromatographic analysis, is unknown for many of these compounds and therefore, a liquid chromatographic method will be used to analyze and quantitate degradation products for this investigation. Many variables exist when considering liquid chromatographic method development including column selection, mobile phase selection, gradient, etc. that are all sensitive to the degradation products for this research. Each parameter will be investigated closely to determine optimal conditions for analysis.

Concentrations for these degradation products are expected to be low considering that most parent product concentrations found in soil are low themselves. Moreover, one parent compound can result in multiple degradation products tending for only a percentage of the parent compound to generate the major degradation product. In consideration of these two factors, it is expected that low concentrations will be found in soil. Wilson *et al.* found concentrations of TCPY, in soils surrounding a day care and residential houses in North Carolina, at concentrations ranging from 0.6 ng/g (ppb) to 111 ng/g (Wilson, Iachen et al. 2002). Since detection limits need to be low to analyze these

concentrations, a tandem mass spectrometry detection technique may be needed to assess exposure. Investigation into MS interface selection and optimal settings for efficient ionization for analysis will be performed for these degradation products.

The method development procedure will be described in the format that it was performed. The mass spectrometry/ionization research was performed first to determine which LC-interface was appropriate for the degradation products and all of the individual parameters needed to efficiently ionize the analyte. Next, the LC development was performed which entails investigation into different factors involved in the sufficient retention and separation for the degradation products. Next, the research detailing the steps followed for successful extraction and clean-up of these degradation products from soil will be described. Lastly, overall method validation requirements will be explained in detail involving method precision, accuracy, analyte recoveries, detection limits, etc. and problems encountered including matrix effects.

Tandem Mass Spectrometry Background and Optimization

Two different ionization techniques were researched for optimal intensity and selectivity for the analytes of interest. All optimization experiments were performed on a Thermo Quantum MS/MS (Waltham, MA).

Atmospheric Pressure Chemical Ionization

Atmospheric pressure chemical ionization is a "soft" ionization process that converts liquid effluent from the column into a gas/vapor discharge from a heated vaporizer sample tube at temperatures up to 600ºC. The nebulized mist is then ionized, under ambient conditions, by a current discharge from the corona needle (up to 100µA). Sample vapor is ionized by ion-molecule reactions with corona needle discharge reagent ion plasma from mobile phase vapor. APCI is a useful ionization because it can be applied to ionic, polar and nonpolar molecules. Below are diagrams that explain the APCI process.

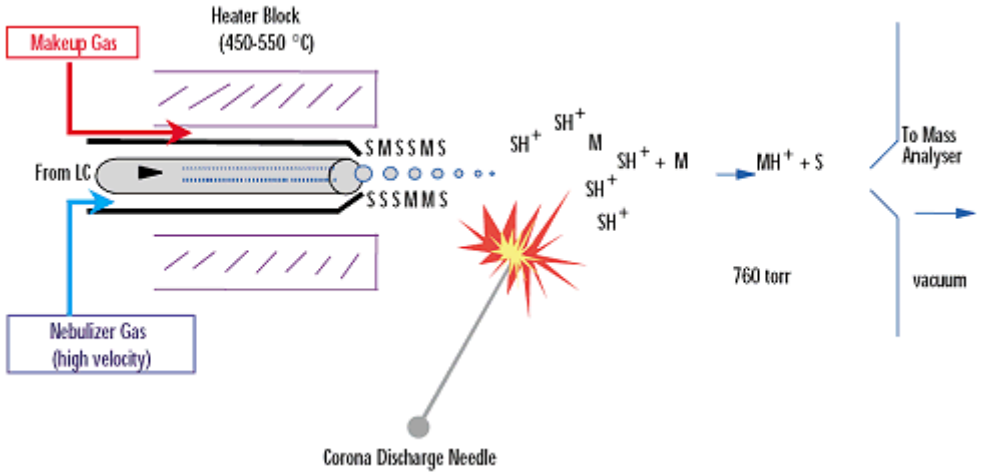


Figure 3.4 APCI Process Schematic *Used with permission* (Mallett 2006).

Electrospray Ionization

Electrospray ionization is another ambient ionization process that ionizes analytes in the liquid phase with a charged capillary (3-5kV) and transfers these ions into the gas phase (Willoughby 2002). The key requirement for successful ESI is that the analytes of interest must exist in solution as an ion (Willoughby 2002). The picture below shows a depiction of the ESI process. The charge transfer mechanism for ESI occurs by one of two potential theories. The charged residue model (CRM) explains the evaporation of solvent from the highly charged droplets exiting the charged capillary as a series of coulombic explosions. A coulombic explosion occurs when the Rayleigh limit is overcome, *i.e.*, evaporation of the solvent causes an abundance of neighboring ions to repel at a greater force than the surface tension of the droplet liquid over that same area (Nguyen and Fenn 2007). The end result is a single solute molecule that retains the charges to become a gas-phase ion (Nguyen and Fenn 2007). The ion evaporation model (IEM) challenged the last part of the CRM model; specifically, it describes how the last charged droplet containing only one solute molecule would be so overcome by charge density on its surface that the, "…resulting field would be sufficient to push one or more of those surface ions into the ambient gas" (Nguyen and Fenn 2007). The diagram below depicts these two theories.

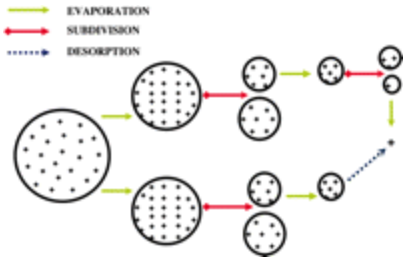


Figure 3.5 Possible Pathways for Ion Formation in ESI *Used with permission* (Nguyen and Fenn 2007).

Electrospray ionization is considered to be an extremely soft ionization mechanism, maintaining the integrity of the molecule being analyzed even large macromolecules with molecular weights greater than $1x10^6$ amu (Willoughby 2002). In addition to soft ionization, ESI can induce multiple charges for a single molecule aiding in determining exact molecular weight measurements for biomolecules (Pramanik 2002). Heated-ESI (HESI) is a variation of ESI that is used for mobile phases with a high amount of aqueous phase and/or at high flow rates. Shown in Figure 3.6 below is the desolvation process starting earlier (1) with the advantage of heating the auxiliary gas in the ESI probe prior to sample heating in the ion transfer tube (2).

Figure 3.6 ESI Heating Chambers *Used with permission* (Thermo 2006).

APCI vs. ESI

Because the degradation products for this study include polar, neutral analytes in addition to polar, ionic species, both APCI and ESI (negative and positive ion modes) ionization

were explored to determine maximum ion efficiency for the analytical method. Typically, ESI is limited to ionic species. However, there have been studies that have reported some neutral species being successfully ionized with ESI with higher efficiencies than APCI (Ternes, Bonerz et al. 2001; Nilsson, Viberg et al. 2006; Vieno, Tuhkanen et al. 2006). In addition to the two difference ionization techniques, several other parameters were investigated to determine maximum ionization efficiency (Table 3.3).

	ESI	H-ESI	APCI
Liquid Flow Rate (µL/min)	✓	✓	✓
Ion Transfer Temperature (°C)	✓	✓	✓
Sheath Gas Pressure (psi/bar)	✓	✓	✓
Auxiliary Gas Pressure (psi/bar)	✓	✓	✓
Spray Voltage (V)	✓	✓	
Tube Lens Voltage (V)	✓	✓	✓
Capillary Temperature (°C)	✓	✓	
Vaporizer Temperature (°C)		✓	✓
Corona Discharge (µA)			✓

Table 3.3 LC-MS/MS Parameters.

Additionally the components of the mobile phase eluant (organic portion, aqueous portion, buffer concentration) from the LC outlet affect these parameters and overall ionization efficiency. Tube lens voltage, collision energy (source collision induced dissociation), collision induced dissociation in Q2 and the temperature parameters (capillary, vaporizer and ion transfer tube) are all analyte dependent.

MS/MS Infusions

Each analyte was infused into the MS/MS with 100% acetonitrile as the mobile phase at concentrations between 2-5ppm (mg/l) to analyze parent-daughter ion pairs appropriate for mass analysis, and the analyte dependent MS/MS parameters (tube lens voltage, temperature parameters) for both APCI and ESI. Once the base peak became apparent (in most cases the molecular weight of the infused analyte +/- 1amu or $[M+H]^+$, $[M-H]^-$), it was chosen for further optimization. Each base peak (if appropriate for that analyte) was further optimized to determine what tube lens offset, temperature parameters resulted in the highest intensity normalized for the mass spectrum. A total ion current (TIC) was measured simultaneously with changes in each parameter to determine intensity levels. The example below shows the fluctuations in intensity associated with increasing the spray voltage, which supplies the potential required to ionize samples, on the ion under investigation (*m/z* 137; 4-nitrophenol parent ion) (Figure 3.7). As shown, the spray voltage is increased steadily where it peaks at ~2500 volts before decreasing in intensity with any higher voltage.

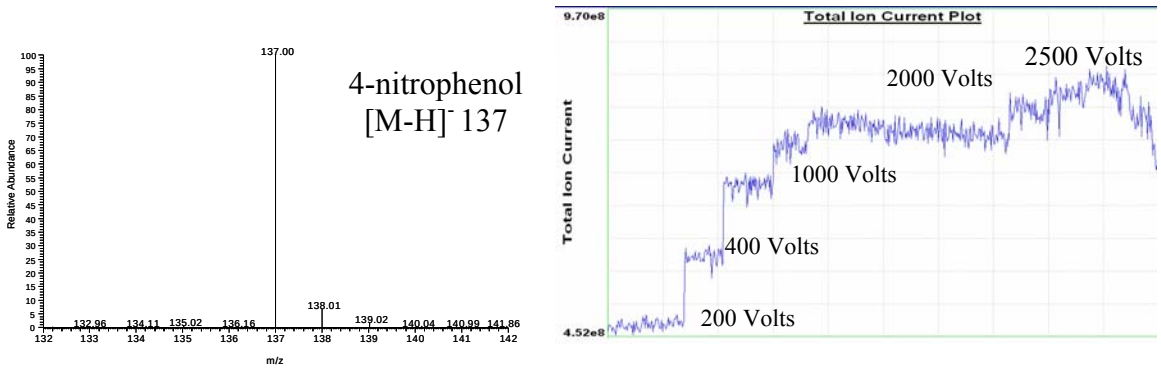


Figure 3.7 Fluctuations in Ion Current with Increasing Spray Voltage in ESI.

Another parameter necessary for MRM analysis is selection of daughter ions in Q3. A scan is performed over a mass range while observing the change to the parent ion after collision energy added. Below are examples of the pyrethroid degradation product, 4F3PBA and a series of different collision energies induced to breakdown the parent ion for 4F3PBA (*m/z* 231) in Q2.

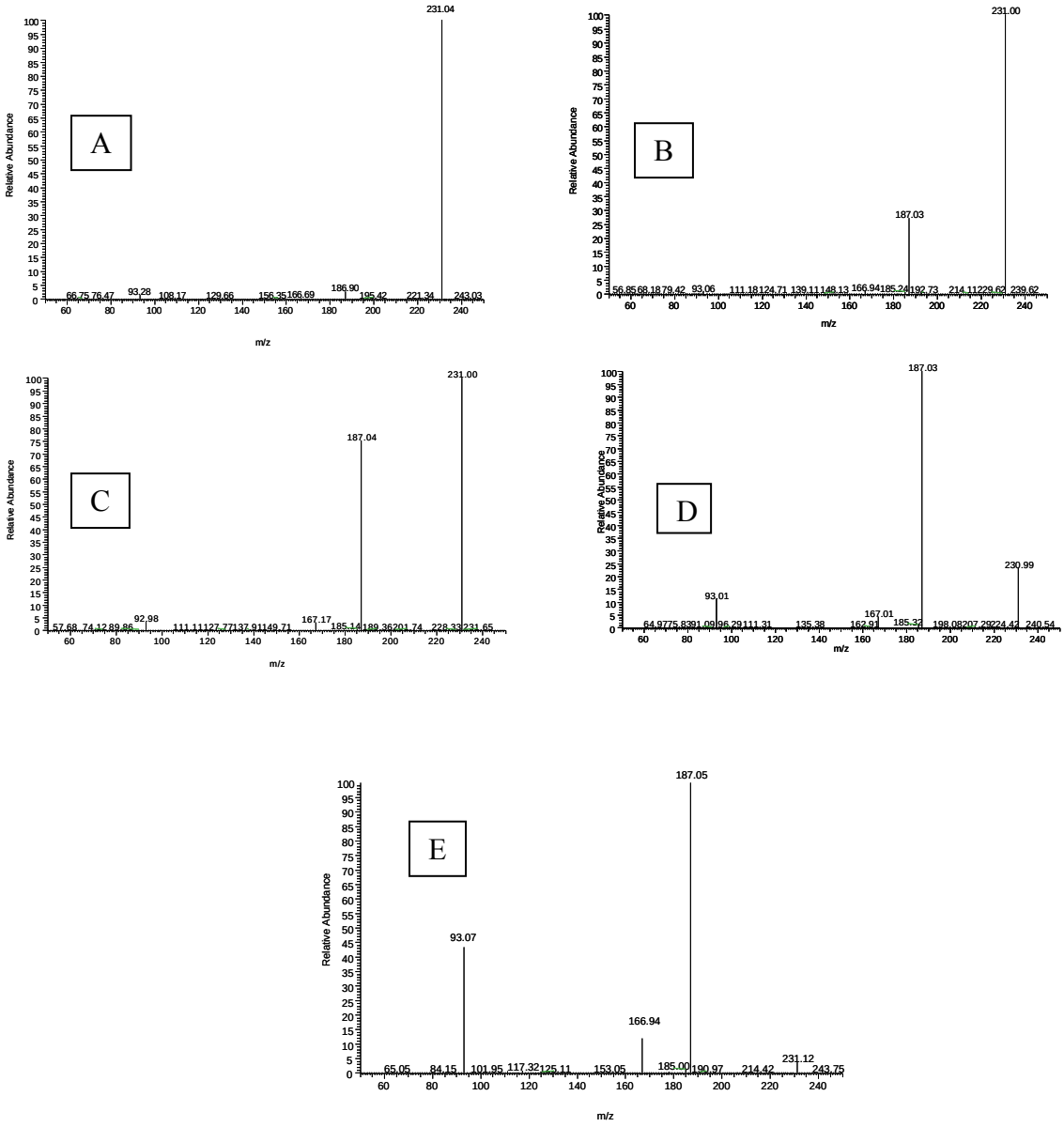


Figure 3.8 Parent-Daughter Ion Formations for 4F3PBA with Various Collision Energies in Q2 in ESI (A-E).

Looking at "A", the parent ion of 4F3PBA (*m/z* 231) is apparent in Q3 because there is no collision energy being applied. In "B", a collision energy of 5 is applied to Q2 to

produce a daughter ion *m/z* 187. In "C", a collision energy of 10 is applied to Q2 producing almost equal amounts of daughter ions as parent ions present in Q3. In "D", the daughter ion *m/z*187 is the dominant ion in Q3 with the parent ion almost completely dissociated. The appearance of a second daughter ion (*m/z* 93) is present either from either further breakdown of the parent ion or the daughter ion (*m/z* 187). In "E", the parent ion is completely dissociated with only daughter ions present. This optimization is investigated as such for each degradation product to find appropriate parent/daughter ion pairs and also to further fine-tune daughter ion parameters as well.

For three of the analytes, phorate sulfone, phorate sulfoxide, and terbufos sulfone, the most abundant ion was a water adducted molecular ion, or a water cluster ion. For example, the spectra of terbufos sulfone (*m/z* 320) and its most abundant ion at *m/z* 338 indicating some water cluster formation during ionization with this analyte, perhaps due to hydrogen bonding (Figure 3.9). This has been reported in the past with various applications (Goebbert, Chen et al. 2006; Mori, Asakawa et al. 2006).

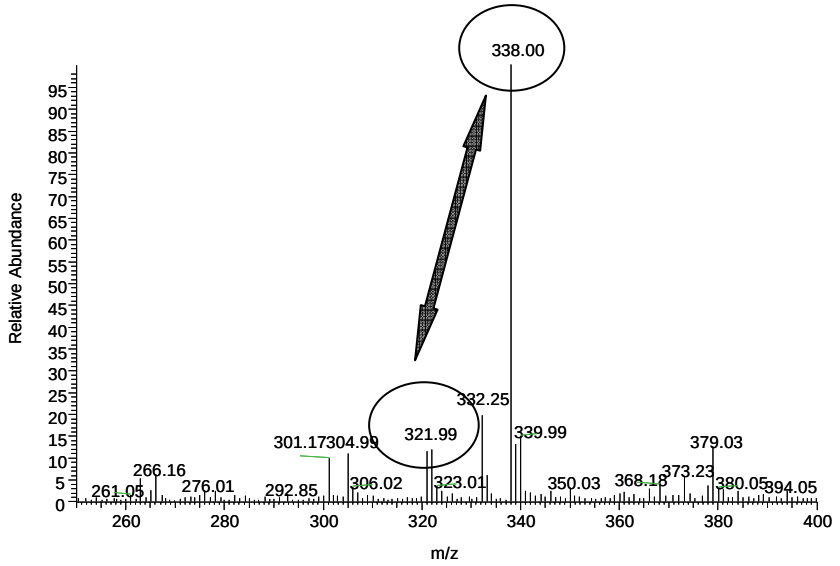


Figure 3.9 MS Spectra of Terbufos Sulfone and Abundant Water Cluster Ion Formation in ESI.

After analyzing all of the analytes in APCI and ESI, the analytes ionized most efficiently with ESI. The pyrethroid breakdown products with MDA and TCPY had extremely poor ionization with APCI. Typically APCI is better suited for neutral analytes or analytes with high proton affinity. The degradation products are ionizable analytes better suited for ESI. The parameters listed below were found to be optimal settings for all individual analytes with ESI.

SELECTED REACTION MONITORING PARAMETERS

QUANTIFICATION ANALYTE	IONIZATION MODE	CID (V)	PARENT ION (m/z)	CE (V)	PRODUCT ION (m/z)
4-NP	NEG	15	138	32	108
4-NP-LABEL	NEG	20	144	38	114
TCPY	NEG	10	198	0	198
TCPY-LABEL	NEG	20	202	0	202
DCCA	NEG	15	207	0	207
DCCA-LABEL	NEG	20	210	0	210
DBCA	NEG	0	343	11	81
DBCA-LABEL	NEG	20	304	28	81
3-PBA	NEG	20	213	40	93
3-PBA-LABEL	NEG	20	219	41	99
CDCA	NEG	10	197	24	97
CDCA-LABEL	NEG	15	204	30	99
4F-3PBA	NEG	15	231	53	93
CFCA	NEG	15	241	37	121
Phorate Sulfone	POS	0	310	17	171
Phorate Sulfoxide	POS	15	277	36	143
Terbufos Sulfone	POS	0	338	10	321
Terbufos Sulfoxide	POS	0	305	12	187
MDA	NEG	0	273	12	141
MDA-LABEL	NEG	0	280	12	147
IMPY	POS	15	153	18	84
IMPY-LABEL	POS	15	157	32	88
DMP	NEG	10	125	49	79
DMP-LABEL	NEG	10	131	47	79
DEP	NEG	15	153	31	79
DEP-LABEL	NEG	15	163	55	79
DMTP	NEG	10	141	35	95
DMTP-LABEL	NEG	0	147	37	97
DETP	NEG	0	169	32	95
DETP-LABEL	NEG	10	179	33	95
DMDTP	NEG	0	157	16	142
DMDTP-LABEL	NEG	10	163	50	79
DEDTP	NEG	10	185	0	185
DEDTP-LABEL	NEG	10	189	30	111

Table 3.4 MS/MS Target Analyte Parameters.

Although most analytes some analytes had higher intensities in the negative mode, some analytes gave better results in the positive mode. Therefore, two separate MS/MS tuning files had to be created because the instrument does not have the capacity to scan ions in both positive and negative modes simultaneously. As a result, each sample will have to be injected twice, a requirement to consider if sample conservation is important. The tuning parameters are detailed in Figure 3.10 below.

Positive Mode

Device	Value
Spray Voltage	3500
Vaporizer Temperature	250
Sheath Gas Pressure	80
Ion Sweep Gas Pressure	0.0
Aux Gas Pressure	10
Capillary Temperature	275
Tube Lens Offset	15
Source CID	0
Collision Pressure	1.5
Collision Energy	-10
Quad MS/MS Bias	-0.1

Negative Mode

Device	Value
Spray Voltage	2500
Vaporizer Temperature	250
Sheath Gas Pressure	70
Ion Sweep Gas Pressure	0.0
Aux Gas Pressure	30
Capillary Temperature	250
Tube Lens Offset	-87
Source CID	0
Collision Pressure	1.5
Collision Energy	18
Quad MS/MS Bias	0.1

Figure 3.10 Tuning Parameters for Negative and Positive Modes in MS/MS.

Additional optimization of these parameters was performed after the mobile phase was selected with liquid chromatography optimization because of potential effects associated with buffer, pH, solvent and the ionization process.

Liquid Chromatography Background and Optimization

Liquid chromatography was chosen as the separation technique for the degradation products due to their polarity and ionizable properties. The nonspecific organophosphate degradation products (dialkylphosphates) have been analyzed by gas chromatography; however, this technique would require a chemical modification to increase volatility that could possibly interfere with recovery and detection limits (Bravo, Caltabiano et al. 2004). To eliminate this obstacle, liquid chromatography can separate these compounds as they exist in solution. Two liquid chromatographic separations were investigated to achieve complete separation of the degradation products. Complete separation is an integral component to quantitate accurate and precise analyte responses. Because of the possibility of many components in the soil matrix having similar molecular weights as the degradation products of interest as shown in Figure 3.11, separation allows for selective quantitation and interpretation of the target analytes without possible interferences.

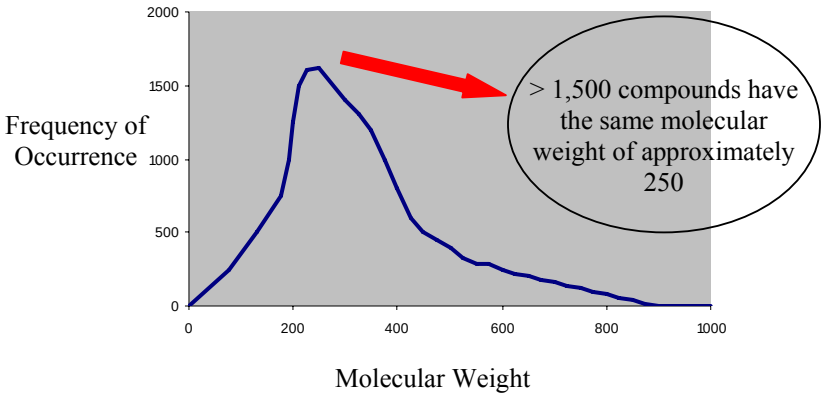


Figure 3.11 Frequency of Occurrence vs. Molecular Weight of Compounds *Adapted from* (Willoughby 2002).

Reverse Phase Chromatography

Reverse phase (RP) chromatography is the most widely used separation technique in analytical methodology(Willoughby 2002). For RP separation, the mobile phase is the polar constituent and the stationary phase is non-polar. The analytes partition between the two phases and are separated based on their intrinsic polarity. Polar and ionic analytes are not retained on the column and elute quickly. However, as the mobile phase transitions to a more non-polar solvent, non-polar analytes elute off the column at separate times. Therefore, RV separation is highly amenable to non-polar or neutral analytes. The most common packing material for RP separations are C-18 polymeric or silica based columns; however, there are many types of columns that are investigated for routine analysis including C8, C2, phenyl, etc. (Willoughby 2002).

Normal Phase Chromatography

Normal phase (NP) chromatography has the opposite configuration as RP chromatography. In NP chromatography, the stationary phase is polar while the mobile phase is non-polar. Polar analytes bind tightly to the stationary phase until the mobile phase becomes more polar for the analytes to elute. NP chromatography has phased out considerably since the 1970s due to poor reproducibility, slow equilibrium times and poor chromatography (tailing/fronting, etc.) (Hemstrom and Irgum 2006). Other methods of separating polar analytes include ion-exchange or ion-pairing. However, these methods are problematic in that they only work with ionizable analytes and ion-pairing can cause MS signal suppression (Grumbach LCGC). In addition, some analytes with extremely low or high pKas require a likewise extreme pH solvent(s) that are not

amenable to chromatography columns or detection systems. Most NP-like chromatography today is with hydrophilic interaction liquid chromatography (HILIC) (Hemstrom and Irgum 2006). Similar to NP chromatography, HILIC chromatography is run on polar stationary phase. HILIC columns can be utilized as silica, amino, diol, polyhydroxyethyl aspartamide and cyclodextrin-based packings (Grumbach, Wagrowski-Diehl et al. 2004). However, unlike classic NP, water is present at much higher amounts (>5%) in the mobile phase with HILIC. In addition, HILIC uses water-miscible solvents like acetonitrile which are more amenable to detection systems rather than the typical water-immiscible solvents like hexane and chloroform found with NP chromatography (Zhou, Song et al. 2005). The mechanism of separation is through partitioning between a layer of mobile phase enriched with water that is partially immobilized on the stationary phase and the hydrophobic mobile phase (Alpert 1990). Since the inception of HILIC chromatography in 1990, most applications have been focused on bioanalytical separations, specifically with carbohydrate analysis and drug discovery (Hemstrom and Irgum 2006). There are very few publications utilizing this chromatography for possible biomonitoring or environmental monitoring purposes. A search from ISI Web of Science for ("HILIC" and "SEPARATION" and/or "CHROMATOGRAPHY"), yielded only three publications that had applications in environmental research. One study focused on the tobacco-specific nitrosamine metabolite in human plasma, one on the analysis of folate metabolites in plasma and the other publication analyzed dichloroacetic acid in drinking water (Garbis, Melse-Boonstra et al. 2001; Dixon, Delinsky et al. 2004; Pan, Song et al. 2004).

Mobile Phase Selection

Solvent selection is as important a parameter to consider as the type of column selected for separation. Peak shape, retention time, functional group specificity, column backpressure, buffer-analyte interactions and detector background signal levels are all parameters directly related to the solvent chosen for separation (Sadek 2002). Water, acetonitrile, methanol and tetrahydrofuran are most frequently used solvents for RP separation (Sadek 2002). Hexane and dichloromethane are typical solvents found for NP separations (Willoughby 2002). HILIC type columns require some form of water-organic binary phase for optimal retention. In addition, buffers are routinely added to mobile phases to control pH. Small changes in pH can have major repercussions on retention and reproducibility of peaks (Snyder 1997). Buffering mobile phases is important, specifically when ionized analytes are present and their neutral/ionized state depends on pH of their surroundings. In addition, most columns require specific pH ranges for optimal retention and to prevent degradation. RP LC carried out with C8 or C18 bonded-phase silica-based columns require pH range from 2-8 (Snyder 1997). Buffers are typically chosen for their pKa value that is closest to the desire pH range of the mobile phase (Sadek 2002). However, it is important to keep in mind that for MS analysis, only volatile buffers should be used (Snyder 1997). In addition, buffer concentration is an important parameter to consider when selecting mobile phase constituents. The need for balance of appropriate buffer concentration is important because too little buffer will not support pH changes to your system; however, too concentrated of a buffer can lead to suppression of ionization in MS analysis (Lagana, Fago et al. 1998; Choi, Hercules et al. 2001). In addition to ion suppression, high organic mobile phases can create problems

with some buffers due to insolubility which can lead to clogging problems, high backpressures and overall system malfunction; this effect will be demonstrated later with the HILIC column. Mobile phase considerations are all dependent on the type of LC separation and analyte physical properties and will be detailed in the following sections.

Degradation Product Chromatography

Many of the products for this study have already been investigated for chromatographic separation. The dialkylphosphates, have been typically separated by gas chromatography in the past for biomonitoring purposes (Reid and Watts 1981; Nutley and Cocker 1993; Aprea, Sciarra et al. 1996; Moate, Lu et al. 1999; Hardt and Angerer 2000; Bravo, Caltabiano et al. 2004). These methods involve a chemical derivitization of the DAP to increase its volatility, a necessary requirement for successful gas chromatography. Derivitization can substantially lengthen sample preparation times and can become cumbersome. For instance, several of these GC methods employ pentafluorobenzylbromide, a powerful lachrymator, as the derivitization agent in a process that requires 15 hours for derivitization (Hardt and Angerer 2000). There have been a few LC methods separating DAPs, however, they only focus on a few of the DAPs or they have high detection limits and low selectivity and sensitivity (Bardarov and Mitewa 1989; Hernandez, Sancho et al. 2002; Hernandez, Sancho et al. 2004). Complete chromatographic separation of (DMP and DEP) and (DMTP and DETP) is mandatory for analysis of these compounds because they share similar fragmentation patterns which could lead to false positives(Hernandez, Sancho et al. 2002). DMP (*m/z* 125) fragments into daughter ions *m/z* 79, 63 and DEP (*m/z* 153) fragments into daughter ions *m/z* 125,

79. Similarly, DMTP (*m/z* 141) fragments to daughter ions *m/z* 95, 63 and DETP (*m/z* 169) fragments to *m/z* 141, 95 (Figure 3.12).

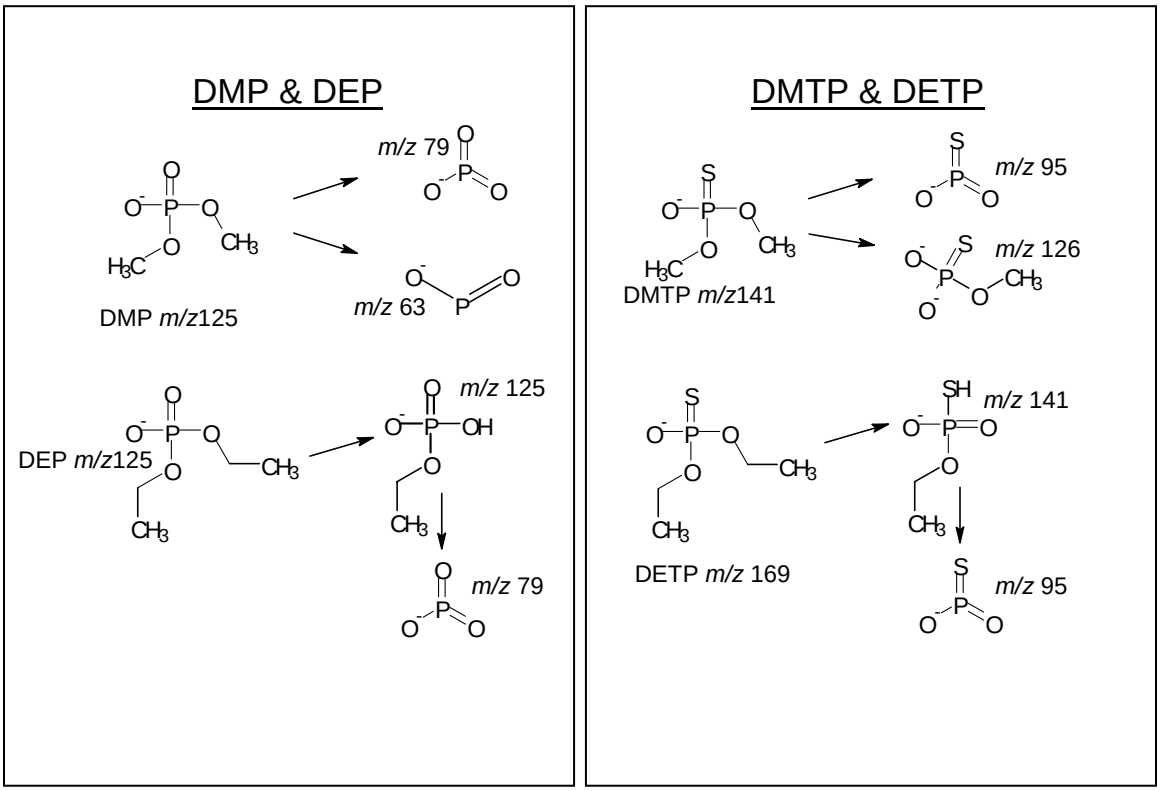


Figure 3.12 Proposed DAP Fragmentation Patterns *Adapted from* (Hernandez, Sancho et al. 2002).

Recently, there was a method published that successfully separated all 6 DAPs in human urine with low detection limits (0.5-1.3 µg/L)(Dulaurent, Saint-Marcoux et al. 2006). Surprisingly, these polar analytes were separated using a reversed-phase LC separation. The specific organophosphate and pyrethroid degradation products have been chromatographically separated together and apart using both GC and LC methods (Leng, Kuhn et al. 1997; Baker, Barr et al. 2000; Sancho, Pozo et al. 2000; Bravo, Driskell et al. 2002; Sancho, Pozo et al. 2002; Olsson, Nguyen et al. 2003). However, all of these analytical methods are for biomonitoring studies in urine and serum and not in any environmental matrix. In addition, a search of the literature with the pesticide classes

used as keywords revealed no analytical methods that combine all three groupings (specific and non-specific OP, pyrethroid degradation products) in a single method. In addition, there are no analytical methods for the determination of CFCA, the bifenthrin and cyhalothrin pyrethroid breakdown product and metabolite or for the sulfones and sulfoxides of phorate and terbufos organophosphorus breakdown products. These are important analytes to monitor because bifenthrin and cyhalothrin are both widely used pyrethroid pesticides used in agricultural and residential settings (Roberts 1999). In addition, the sulfone and sulfoxide products of phorate and terbufos have been shown to have extremely long half-lives (Kamrin 1997).

Both reverse-phase and HILIC chromatography will be investigated for the most complete chromatographic separation for all of the proposed degradation products using an Agilent LC 1100 series and quaternary pump system (Foster City, CA). For reverse-phase research, the Inertsil ODS3 C18 column from GL Sciences (Tokyo, Japan) will be utilized as was used in the Dularent method to determine if this method could be expanded to include the specific OP and pyrethroid degradation products. The ZIC-HILIC column from SeQuant (Southborough, PA) will also be investigated to research separation mechanisms similar to NP chromatography. The HILIC separation mechanism provides an additional retention mechanism that may be ideal for the group of degradation products to be analyzed. There are secondary electrostatic interactions due to the sulfobetaine zwitterionic silica-based stationary phase in addition to the hydrophilic partitioning mechanism (Figure 3.13). This additional interaction may be favorable for the degradation products of interest because many of these products will be

in a charged state in addition to the polar characteristic that is successful with HILIC separations.

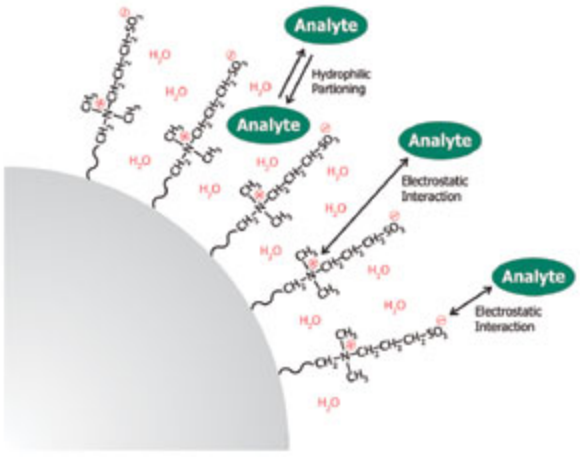


Figure 3.13 HILIC Interactions *Used with permission* (SeQuant 2006).

Inertsil Chromatography

As with most RP chromatography, investigating various portions of water in the mobile phase to determine retention will be carried out with the degradation products utilizing the Inertsil separation column. A RP separation for this group of analytes will be challenging as most of the degradation products are ionic within the column's working pH range (pH2-8) (see Table 3.5). All injections were made with a flow rate of 100µL/min.

Physical Properties of Degradation Products													
				pH3 (25ºC)		pH4 (25ºC)		pH5 (25ºC)		pH6 (25ºC)		pH7 (25ºC)	
	ANALYTE	MOLECULAR WEIGHT	pKa[a]	Charge State[b]	log D	Charge State[b]	log D	Charge State[b]	log D	Charge State[b]	log D	Charge State[b]	log D
PYRETHROID PRODUCTS	DBCA(*trans/cis*)	297.98	3.91	Partially ionized	3.04	Partially ionized	2.74	Partially ionized	1.97	Ionized	1.01	Ionized	0.10
	3PBA	314.22	3.82	Partially ionized	2.93	Partially ionized	2.59	Partially ionized	1.79	Ionized	0.83	Ionized	-0.07
	4F3PBA	232.21	3.99	Partially ionized	3.09	Partially ionized	2.82	Partially ionized	2.08	Ionized	1.12	Ionized	0.20
	CFCA	242.63	3.80	Partially ionized	2.27	Partially ionized	1.93	Partially ionized	1.11	Ionized	0.15	Ionized	-0.74
	CDCA	198.22	4.09, 4.83	Partially ionized	1.43	Partially ionized	1.18	Partially ionized	0.15	Ionized	-1.63	Ionized	-3.54
	DCCA(*trans/cis*)	209.07	3.89	Partially ionized	2.44	Partially ionized	2.13	Partially ionized	1.35	Ionized	0.39	Ionized	-0.52
SPECIFIC ORGANOPHOSPHATE PRODUCTS	IMPY	153.01	1.51, 8.14, 10.21	Neutral	2.84	Neutral	2.85	Neutral	2.86	Neutral	2.85	Neutral	2.83
	MDA	274.30	2.49, 6.27	Partially ionized	1.05	Partially ionized	0.79	Ionized	-0.03	Ionized	-1.41	Ionized	-3.20
	4-NITROPHENOL	139.11	8.07	Neutral	-0.44	Neutral	-0.44	Neutral	-0.45	Neutral	-0.45	Partially ionized	-0.48
	356 TCPY	198.40	10.02	Neutral	3.07	Neutral	3.07	Neutral	3.07	Neutral	3.07	Neutral	3.07
	P. SULFONE	292.36	n/a	Neutral	1.90	Neutral	1.90	Neutral	1.90	Neutral	1.90	Neutral	1.90
	P. SULFOXIDE	260.30	n/a	Neutral	0.97	Neutral	0.97	Neutral	0.97	Neutral	0.97	Neutral	0.97
	T. SULFONE	320.41	n/a	Neutral	2.94	Neutral	2.94	Neutral	2.94	Neutral	2.94	Neutral	2.94
	T. SULFOXIDE	304.42	n/a	Neutral	2.45	Neutral	2.45	Neutral	2.45	Neutral	2.45	Neutral	2.45
NON-SPECIFIC ORGANOPHOSPHATE PRODUCTS (DAPs)	DMP	126.05	2.00	Partially ionized	-1.19	Ionized	-1.99	Ionized	-2.36	Ionized	-2.43	Ionized	-2.44
	DEP	154.10	1.95	Partially ionized	-0.55	Ionized	-1.34	Ionized	-1.69	Ionized	-1.75	Ionized	-1.75
	DMTP	142.12	2.86	Partially ionized	0.19	Ionized	-0.58	Ionized	-1.34	Ionized	-1.65	Ionized	-1.70
	DETP	170.17	2.86	Partially ionized	0.87	Ionized	0.11	Ionized	-0.65	Ionized	-0.96	Ionized	-1.01
	DMDTP	158.18	1.35	Ionized	0.23	Ionized	0.09	Ionized	0.08	Ionized	0.08	Ionized	0.08
	DEDTP	186.24	1.35	Ionized	0.92	Ionized	0.78	Ionized	0.76	Ionized	0.76	Ionized	0.76

[a] Calculated from ChemAxon© (Other minor pKas may exist but are negligible to this research)

[b] Estimated based on pKas

Log P estimates

Table 3.5 Chemical and Physical Properties of Degradation Products.

Theoretically, RP chromatography successfully separates and retains non-polar and/or neutral compounds. The sulfones and sulfoxides in this analysis should therefore retain quite well using RP separation. Also in theory, neutralizing the ionized analytes in the group would be desirable for better retention. Because the ionizable analytes are all acids at normal pH ranges, bringing the pH of the mobile phase 2 units below the analyte pKa should effectively neutralize these acids. However, the DAPs have very low pKas (1.35-2.6) that would require extremely acidic conditions for neutralizing, a situation that would be highly unfavorable to the column and to the detection system. Nevertheless, Dulaurent *et al*. managed to completely separate the DAPs using a mobile phase with a pH of 3 (Dulaurent, Saint-Marcoux et al. 2006). The beginning of RP separation for these analytes will start by following the example of Dulaurent *et.al*., with a mobile phase set at pH3 (2mM ammonium formate buffer).

First, the reproduction of the method published by Dulaurent *et.al.,* was performed with the DAPs. The mobile phase employed in this method was a linear gradient from 30% acetonitrile in water to 90% acetonitrile in water (pH3). Interestingly, the DAPs did not separate successfully (DMP and DEP co-eluted) when reproducing the LC conditions specified in the Dulaurent publication. Only when acetonitrile was replaced with methanol did the analytes successfully separate. For that reason, the RP evaluation with the Inertsil column was continued using methanol as the organic portion of the mobile phase.

The other degradation products were injected into the LC to determine retention with varying amounts of water in the mobile phase (using HESI for detection). The initial goal was to observe retention shifts with 10% increments of the water content in the mobile phase at pH3 (using formic acid to decrease to pH3). However, after several injections, it became increasingly obvious that several of the analytes had poor retention on the RP column. The following table is a list of changing retention times up to 50% water in methanol highlighting the analytes with poor retention.

RP Inertsil ODS-3 Column at pH3 RETENTION TIME SHIFTS

Analyte	90 MeOH/ 10dH2O	80 MeOH/ 20dH2O	70 MeOH/ 30dH2O	60 MeOH/ 40dH2O	50 MeOH/ 50dH2O
TCPY	2.59	2.93	4.52	7.86	12.43
4-NP	1.74	1.92	2.35	2.87	2.92
IMPY	1.71	1.78	1.94	1.92	1.82
MDA	2.60	2.96	2.90	2.86	2.82
CFCA	3.24	4.55	5.19	11.80	28.80
3PBA	2.28	2.78	4.77	9.71	10.60
4F3PBA	2.42	2.97	4.98	10.55	11.31
trans- DCCA	2.03	2.58	4.25	7.79	13.31
cis- DCCA	2.08	2.81	4.84	9.31	20.43
CDCA	1.73	1.93	2.33	2.91	7.35/10.69
trans-DBCA	2.13	2.93	5.12	10.58	21.46
cis- DBCA	2.15	3.05	5.62	11.67	24.91
DMP	1.25	1.31	1.31	1.55	1.80
DEP	1.26	1.40	1.41	1.75	2.15
DMTP	1.24	1.28	1.30	1.60	1.88
DETP	1.35	1.36	1.63	2.01	2.42
DMDTP	1.35	1.35	1.61	1.73	2.03
DEDTP	1.36	1.38	1.71	2.26	2.67
T Sulfone	3.95	6.21	10.84	16.75	20.26
T Sulfoxide	2.49	3.97	7.54	14.80	18.91
P Sulfone	1.97	2.34	5.71	8.12	11.24
P Sulfoxide	1.64	2.11	4.89	7.04	9.52

Table 3.6 Retention Times with Inertsil Column at pH 3.

4-NP, IMPY, MDA and DAPs

The analytes highlighted in yellow, 4-NP, IMPY, MDA, show poor retention with this RP column as evident with very short retention times in the column and no extended time in correlation with mobile phases with additional water. In addition, the DAPs showed poor retention with mobile phases containing <50% water. It was assumed that since the DAPs retained on the RP column at pH3 with a linear gradient, according to Dulaurent, that perhaps the other degradation products, with even higher pKas, should as well. However, for 4-NP, IMPY and MDA, this was not the case. This questioning result prompted investigation into other possible mechanisms for retention of the DAPs with the RP column. Another key function of retention is partitioning between stationary and mobile phases. Perhaps the analyte, even in its ionized form, could partition with the

stationary phase contributing to retention to a RP stationary phase. Partition coefficients (log P) and distribution coefficients (log D) were calculated for each degradation product to theoretically understand how the analyte behaves (even in the ionized state) between organic and aqueous phases using a computer program for calculations (ChemAxon© MarvinSketch version 1.4.6). These computer derived calculations are attached in Appendix 3A.

A general rule in analytical chemistry is that a neutral analyte is more hydrophobic and therefore more soluble in organic solvents and a charged species is more soluble in aqueous solvents. These accepted rules are based on theoretical calculations such as the partition coefficient (P or K_{ow}) which is a constant derived from the following equation:

$$\text{Partition Coefficient, } P = [\text{Organic}] / [\text{Aqueous}] \text{ Where } [\ ] = \text{concentration}$$

$$\text{Log } P = \log_{10} (\text{Partition Coefficient})$$

Typically a Log P value closer to "1" would indicate that the species would partition toward the organic phase; a value of "0", the species tends to equilibrate between organic and aqueous phases and a value of "-1" indicates that the species would tend to partition with the aqueous phase (Sangster 1997). The distribution coefficient, "D", is used when a species has more than one chemical form (Harris 1999). The following equations are used to describe distribution coefficients:

$$\text{Distribution Coefficient, } D = [\text{Unionized}]_{(o)} / [\text{Unionized}]_{(aq)} + [\text{Ionized}]_{(aq)}$$

$$\text{Log } D = \log_{10} (\text{Distribution Coefficient})$$

In addition to pKa values, understanding the partition/distribution coefficients can aid method validation by giving the analyst some general guidelines to follow.

Looking again at some of the degradation products that have poor retention with the RP column, 4-nitrophenol, although neutral at pH3, has a log D value of -0.44 which would indicate a tendency to gravitate toward the aqueous portion of the mobile phase. This would help explain its poor retention for RP separation. However, this would not explain the poor retention seen with MDA (log D 1.05) and IMPY (log D 2.84). Interestingly, four of the six DAPs have log D values that indicate a tendency to partition toward the organic phase (0.19-0.92). This would help explain the success of Dulaurent *et.al.,* in separating the DAPs on a RP column, even when the DAPs are partially ionized. In addition, the extremely low log D values of DMP, -1.19, would help explain the difficulty of retaining this analyte on a RP column as described in a previous publication (Hernandez, Sancho et al. 2002).

CDCA

The blue highlighted row shows the co-elution of two CDCA isomers until the organic portion is reduced to 50% as shown in Figure 3.14 below.

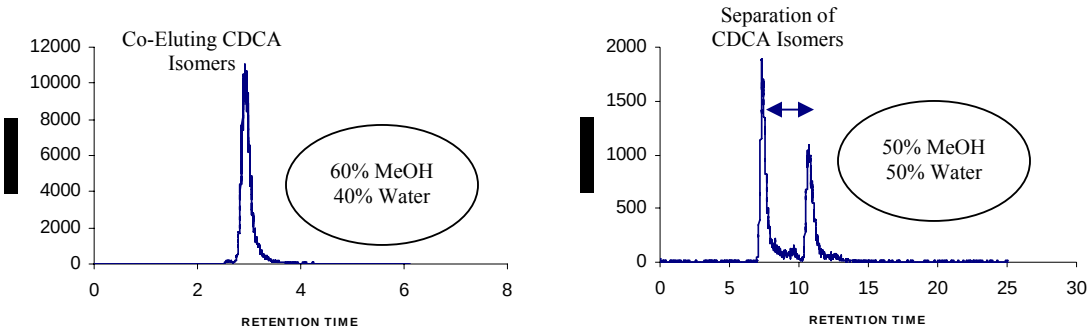


Figure 3.14 CDCA Isomeric Separation.

Although partially ionized at pH3, CDCA has a logD value of 1.43 signifying a high degree of partitioning toward organic phases. Similar to the isomers of DBCA and DCCA, the CDCA isomers are able to be retained on a RP column, an advantage to method development. However, due to poor retention exhibited by the discussed analytes, HILIC separation was investigated next as an alternative. Because HILIC is more appropriate for polar and ionizable compounds, it was expected that this would improve retain for the degradation products not retained with RP chromatography. Conversely, it was also expected that the neutral analytes, the sulfones and sulfoxides would retain less with HILIC separation.

HILIC Chromatography

Typical mobile phases for HILIC consist of 40-97% acetonitrile in water (SeQuant 2006). In addition, HILIC separations are 10-1000 fold more sensitive than RP-LC for polar analytes (SeQuant 2006). For HILIC separation for this research, a series of injections was made for individual degradation products with varying water/acetonitrile portions as well as buffer strength. Injections were made at ~pH7 to promote ionization for the degradation products, a parameter that will enhance retention with the HILIC column (SeQuant 2006). All injections were made with a flow rate of 100µL/min. Table 3.7 below shows retention time behavior for the degradation products with varying amounts of water.

ZIC-HILIC Column at ~pH7 RETENTION TIME SHIFTS

Analyte	70/30 dH_20	85/15 dH_20	87/13 dH_20	95/5 dH_20
cis- DBCA	1.64*	2.2*	2.34*	4.97
trans- DBCA	1.65*	2.21*	2.34*	5.93
CDCA	2.15	4.80	10.76	27.71
cis- DCCA	1.66*	2.21*	2.26*	4.99
trans- DCCA	1.66*	2.22*	2.25*	5.85
4F3PBA	1.59	2.23	2.50	9.47
3PBA	1.62	2.16	2.36	9.06
CFCA	1.60	2.12	2.18	5.10
MDA	2.08	6.20	18.00	36.99
IMPY	2.03	2.47	2.36	3.87
356 TCPY	1.62	2.12	2.24	7.23
4NP	1.81	2.21	2.14	4.12
P Sulfone	<1	<1	<1	3.03
P Sulfoxide	<1	<1	1.94	3.15
T Sulfone	<1	<1	<1	3.01
T Sulfoxide	<1	<1	1.81	3.09
DMP	6.78	8.85	13.75	42.80
DEP	3.95	5.25	9.91	24.70
DMTP	3.18	4.33	7.84	14.15
DETP	2.24	3.04	5.12	10.99
DMDTP	2.22	3.02	4.25	6.50
DEDTP	2.19	2.47	3.07	4.55

* isomers coelute

Table 3.7 Retention Times for HILIC Separation at pH 7.

As expected, the retention time pattern is the opposite of what was found for the Inertsil separations. With increasing portions of water added to the mobile phase for RP-LC, the degradation products retained to the column longer. The pattern is reversed with HILIC-LC; the less water in the mobile phase-the longer the retention for the degradation products. Complete separation was observed for isomeric compounds only with low water contents in the mobile phase (DCCA, DBCA). Interestingly, CDCA isomers were not separated as seen with RP separation. Possibly, isomer-column interactions are more a function of polarity than of ionic or electrostatic interactions. As expected, the neutral sulfones and sulfoxides (highlighted in yellow) had no retention with the HILIC column

until water portions were ≤10% of the mobile phase. Hypothetically, an increase to 97% acetonitrile in water mobile phase may increase retention of these analyte considerably. Although the manufacturer advises use of HILIC columns with at least 3% water in the mobile phase that would enable investigation with a 97% acetonitrile separation; under practical investigation, a mobile phase with <5% water created reproducibility problems. Therefore, the mobile phase chosen for method development and validation was the 95%-5% acetonitrile in water. Mobile phases with buffers consisting of ammonium formate or ammonium acetate are generally advised for HILIC separations with ionic analytes (SeQuant 2006). The presence of buffer is necessary as shown below in order for electrostatic interactions to occur between stationary phase and the analytes. No buffer present in the mobile phase decreases retention dramatically as evident in Figure 3.15 (compared to Figure 3.16) below with 5mM ammonium formate added to the mobile phase. An increase in buffer concentration had no effect on resolution and because of the high organic content of the mobile phase selected, any concentration above 20mM ammonium formate facilitated precipitation. Therefore, 5mM ammonium formate buffer was chosen for method development and validation.

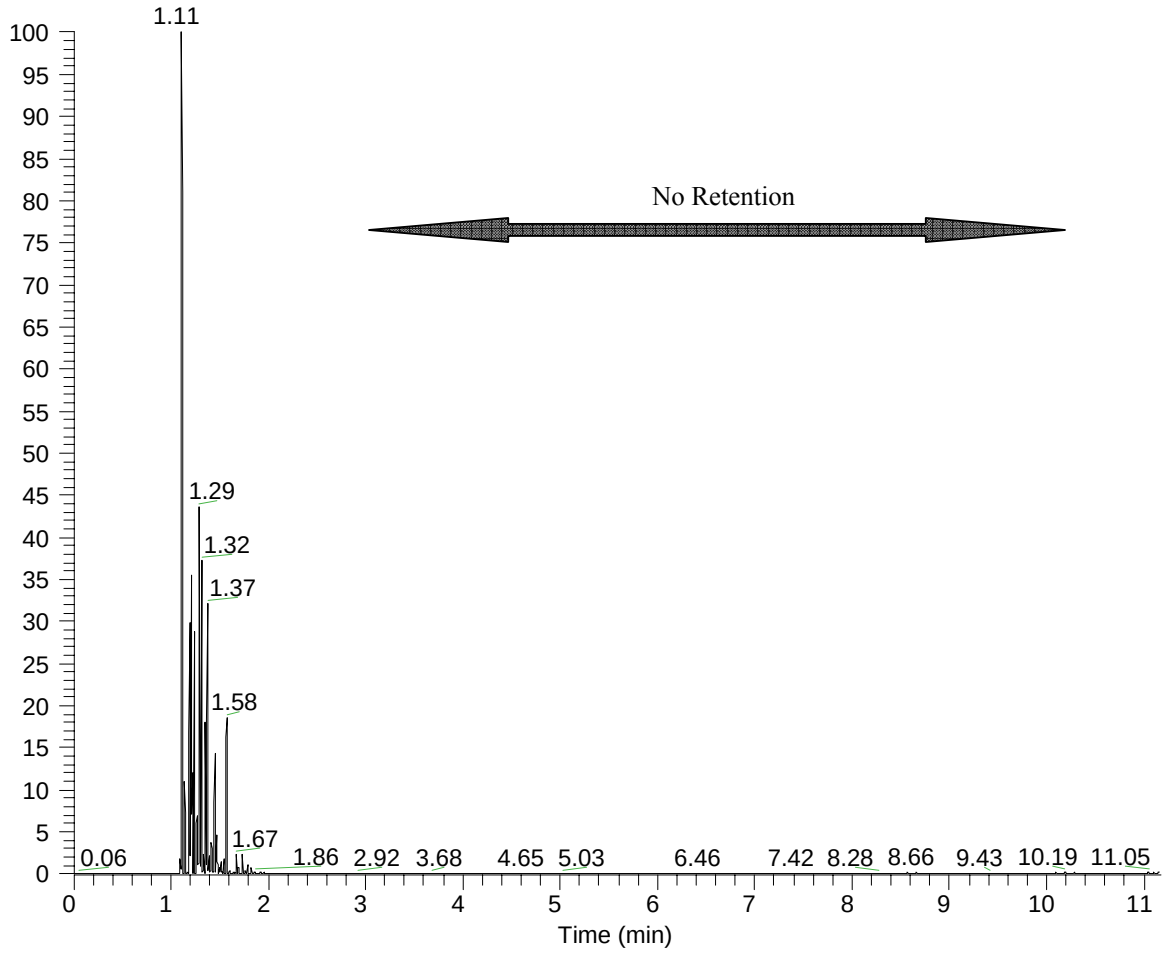


Figure 3.15 TIC of Degradation Products with No Buffer in Mobile Phase.

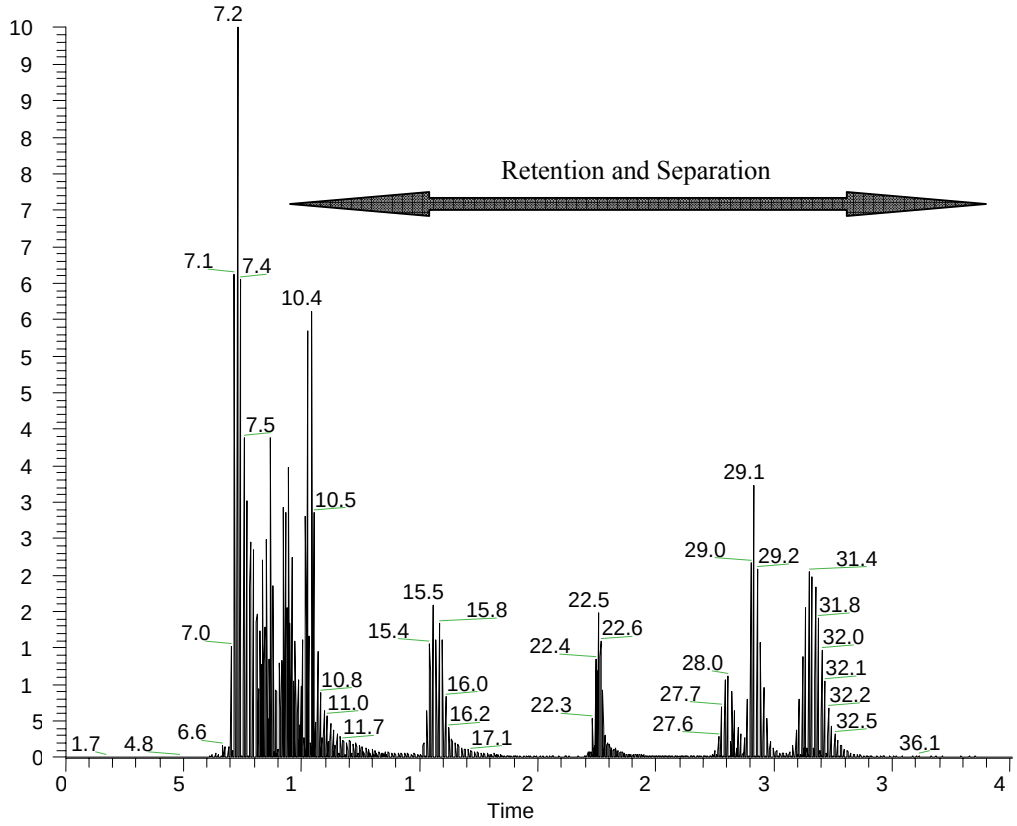
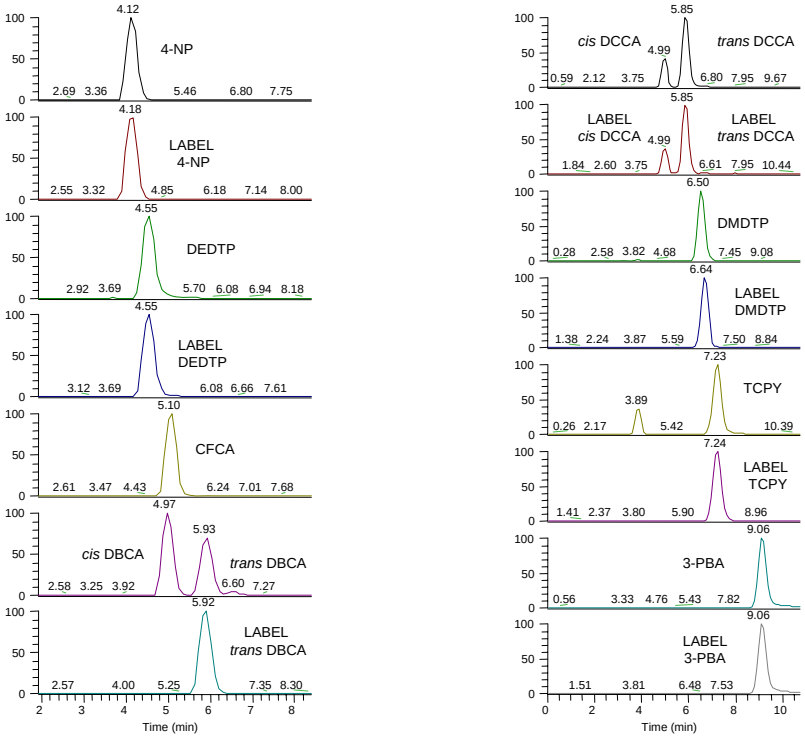


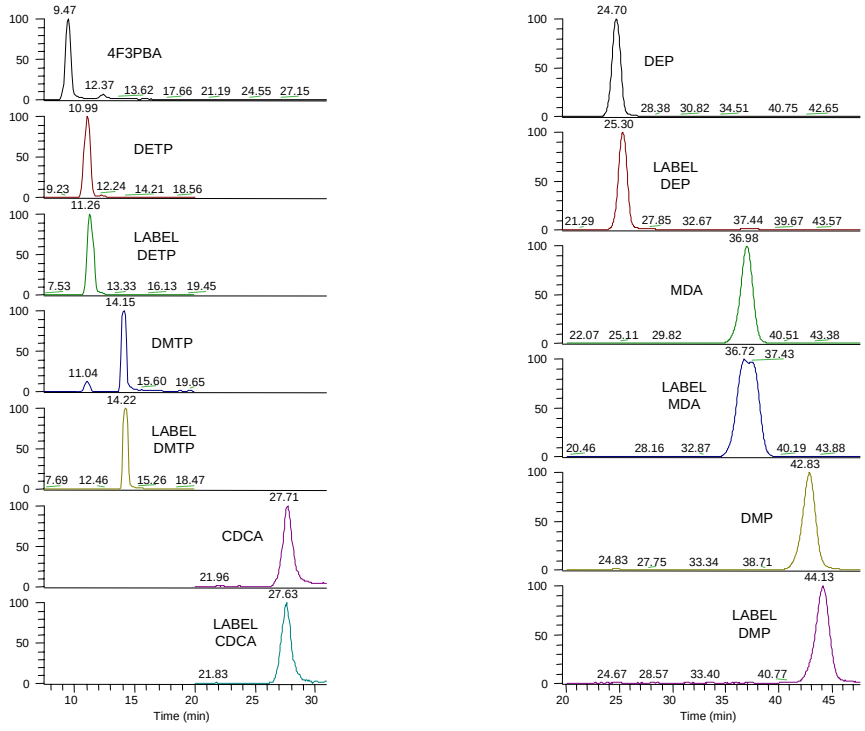
Figure 3.16 TIC of Degradation Products with 5mM Ammonium Formate in Mobile Phase.

Figure 3.17 below show the complete separation of all degradation products and their corresponding label internal standards extracted in 1g of soil at 100ng/g. Chromatograms are resulting SRM ions selected for individual analytes with mobile phase of 95% acetonitrile in 5% water (5mM ammonium formate).

Negative Ions



Negative Ions



Positive Ions

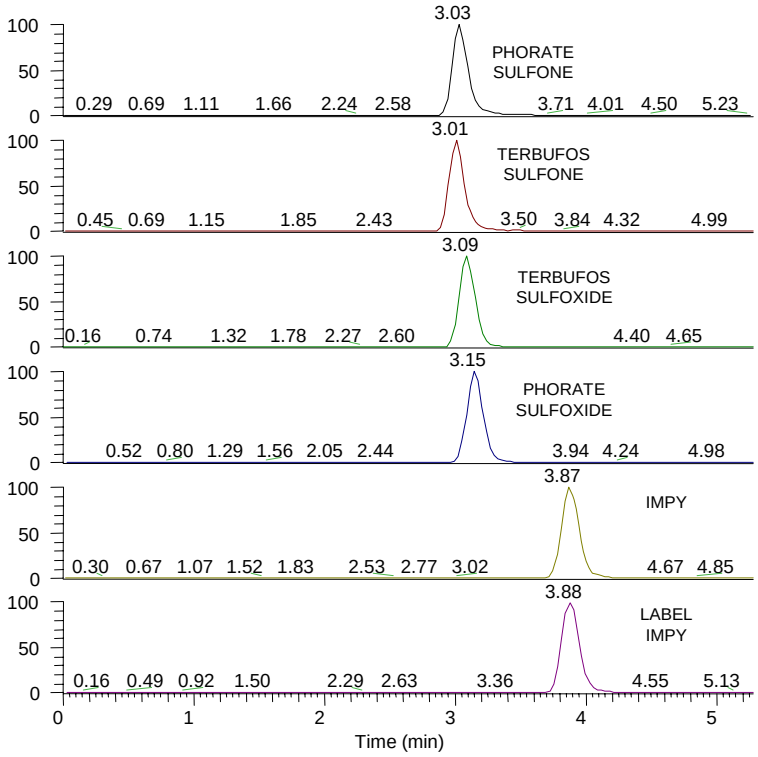


Figure 3.17 SRM of Degradation Products Separated by Mass Filter.

Sample Preparation – Ion Exchange Solid Phase Extraction

"The highest level of uncertainty associated with analytical method development will come from the sample matrix" (Willoughby 2002). The complexity of the targeted matrix for study can create unexpected problems for an analytical method such as matrix effects including ion suppression and/or enhancement in the detection which can lead to false positives and negatives. Co-extracts eluting at a similar time to an analyte of interest would be one example of a false positive. Tandem mass spectrometry should limit this problem because it increases selectivity due to dual mass filtration (Taylor 2005). In addition, including label internal standards instead of chemical analogs close in physical properties of the targeted analytes will help minimize suppression/enhancement issues (Taylor 2005). Fortunately, there is a label isotope standard for most analytes included in this study (excluding CFCA, 4F3PBA, phorate sulfone, phorate sulfoxide, terbufos sulfone, terbufos sulfoxide) which will help improve accuracy of the method. For the analytes lacking internal standard, the label internal standard with the closest chemical and physical properties will be chosen as surrogate to reduce method inaccuracy. Even with tandem MS instrumentation and label internal standards to help compensate for matrix effects, problems can occur that can greatly alter accuracy and precision of the analytical method and increase susceptibility of the instrument to contamination problems. Unknown compounds can be co-extracted with the target analytes and elute later on the LC column. With multiple runs, these co-extracts can build up on the LC column and change column behavior over time (Hopfgartner and Bourgogne 2003). If nonvolatile co-extracts exist in a sample containing target analytes, they may not be readily ionized resulting in decreased ionization efficiency and can dirty

or even contaminate MS hardware (Taylor 2005). Unfortunately, ESI is more susceptible to matrix effects than APCI because in APCI, there is no competition between analytes entering the gas phase; gas phase ion transfer from mobile phase containing targeted analytes is vaporized by a heated corona needle (Bruins, Jeronimus-Stratingh et al. 1999; Jessome and Volmer 2006). At sample concentrations >10-5M, ESI linearity and MS sensitivity can also be decreased substantially by competing co-extracts for charge and space (Tang, Page et al. 2004; Jessome and Volmer 2006). Also, charge states of target analytes can be altered due to co-eluting basic compounds and deprotonation reactions changing their ionization state (Jessome and Volmer 2006). Not only can endogenous compounds be extracted with the target analytes but also exogenous compounds as well from sample preparation materials used to clean samples prior to analysis, for example, polymer coatings in extraction cartridges (Jessome and Volmer 2006). As stated before in the MS section of this thesis, optimized MS settings and parameters must be re-evaluated after introducing the matrix component into the system. Elimination of all possible co-extracting interferences is near impossible. However, the goal of sample preparation is to provide the cleanest extract possible to achieve high accuracy, good precision and sensitivity and to help maintain the analytical instrument. Sample preparation is a strategic undertaking that is a delicate balance of minimizing interfering matrix effects while retaining target compounds.

Soil Sample Preparation

There is a broad range of applications from which to extract chemicals from soil. Probably the most common method is to extract the solid matrix with a solvent

compatible to the target analytes through some form of agitation (shaking, Soxhlet, microwave-assisted, etc.). A detailed discussion of extraction procedures for neutral organic analytes has been provided in the "Parent Pesticide Extraction" discussion. Extraction of polar and/or ionic analytes follows the same pathway as that of their neutral counterparts except the initial extraction solvent would be an aqueous mixture compatible to the target analytes followed by sample clean-up. Because most of the target analytes for this research are ionizable, the possibility of ion-exchange SPE is an option for soil extract clean-up and will be investigated for sample pre-treatment prior to analysis.

Ion Exchange SPE Method Development

SPE has multiple purposes for sample cleanup. Not only does it improve selectivity of a method by aiding in the removal of possible matrix interferences, it pre-concentrates the soil-extracted sample for higher sensitivity and lower detection limits. Additionally, SPE is useful for solvent exchange to more organic when working with aqueous extracts as could be the case with initial soil extraction. SPE methods for acidic compounds are refined to strong ion exchange and weak anion exchange. Determining which method to use is based on pKa knowledge of target analytes. Strong anion exchange SPE sorbents consist of quaternary amine groups permanently charged over pH range of 0-14 (Hennion 1999). Alternatively, weak anion exchange are made from primary and secondary amine groups and do not exhibit permanent charged particles on their sorbent over pH range 0-14 but can be altered as a result of their environmental pH (Hennion 1999).

Polymeric sorbents are preferred over typical silica chain based SPE cartridges for these applications because silica based sorbents are limited to pH range 3-9 and tend to have lower binding capacity than that of the polymer based sorbents (Hennion 1999). Although this range is acceptable and most likely the range for which the target analytes in this research will be investigated, the higher binding capacity in polymeric sorbents are necessary for soil extraction due to possible excess inorganic ions present in soil matrices. A problem for environmental matrices and ion extraction mechanisms exists due to intrinsic inorganic ions that can lead to SPE sorbent overload (Hennion 1999). Therefore, with polymeric sorbents, the possibility of analyte break-thru is reduced and enrichment of the target analytes may be more significant (Chen, Wang et al. 2006).

The first step in developing a clean-up strategy for these analytes in soil is to optimize the SPE portion without matrix components to focus specifically on the selectivity of these analytes for the SPE cartridge retention mechanism. Two ion exchange SPE cartridges were selected for investigation, Phenomenex Strata™ X-AW (Torrance, CA) and Waters® Oasis® WAX (Milford, MA). Both SPE cartridges are mixed mode (hydrophobic and ion exchange capacities) polymeric poly (styrene-divinylbenzene) based cartridges that allows simultaneous retention of both acidic and neutral analytes. The difference between the two sorbents is in the surface polymer used for retention. The Waters WAX cartridge consists of a more complex backbone of multiple aromatic structures cross-linked with more nitro-aromatic compounds than the single benzene structure found with the Phenomenex Strata X-AW cartridge. This difference could have a potential impact on some of the more aromatic analytes (3PBA, 4F3PBA, IMPY, etc.).

Figures 3.18-3.21 below describe the ionization state abundance of the sorbent at particular pH environments.

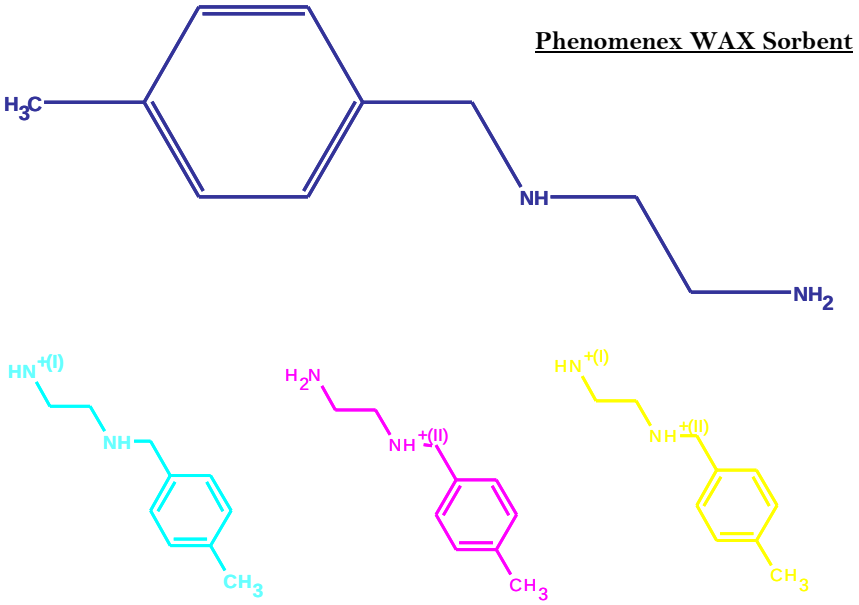


Figure 3.18 Structures of Active Compounds in Phenomenex WAX SPE Sorbent.

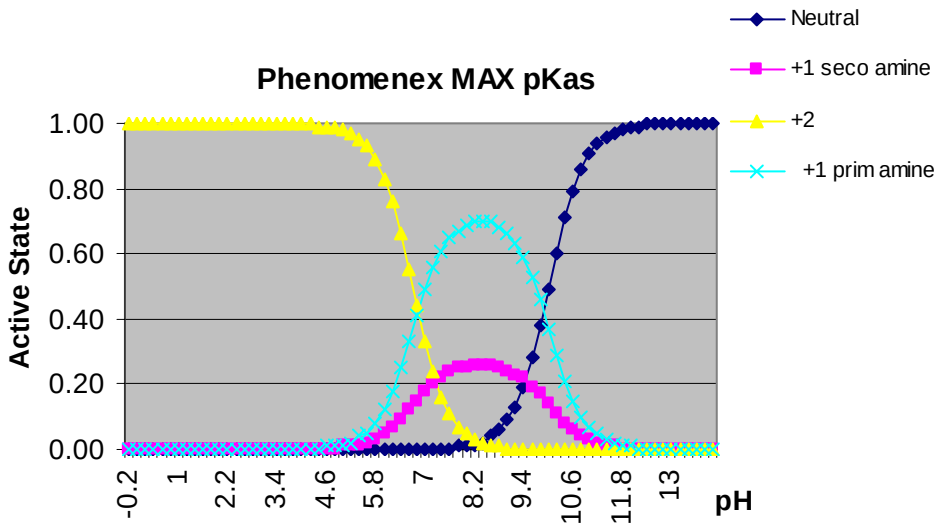


Figure 3.19 Phenomenex WAX Active State According to pH (color matches above structures).

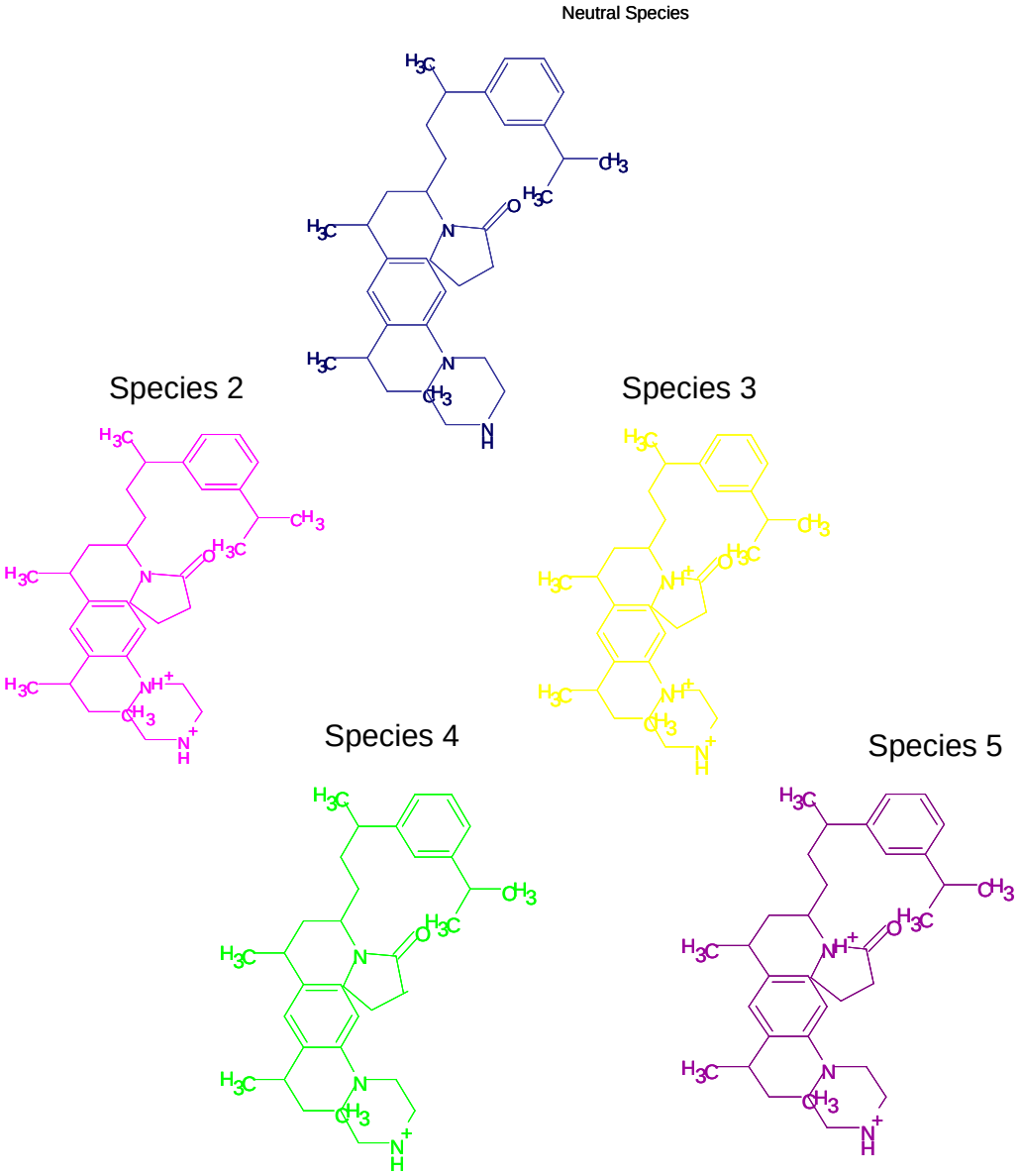


Figure 3.20 Structures of Active Compounds in Waters OASIS SPE Sorbent.

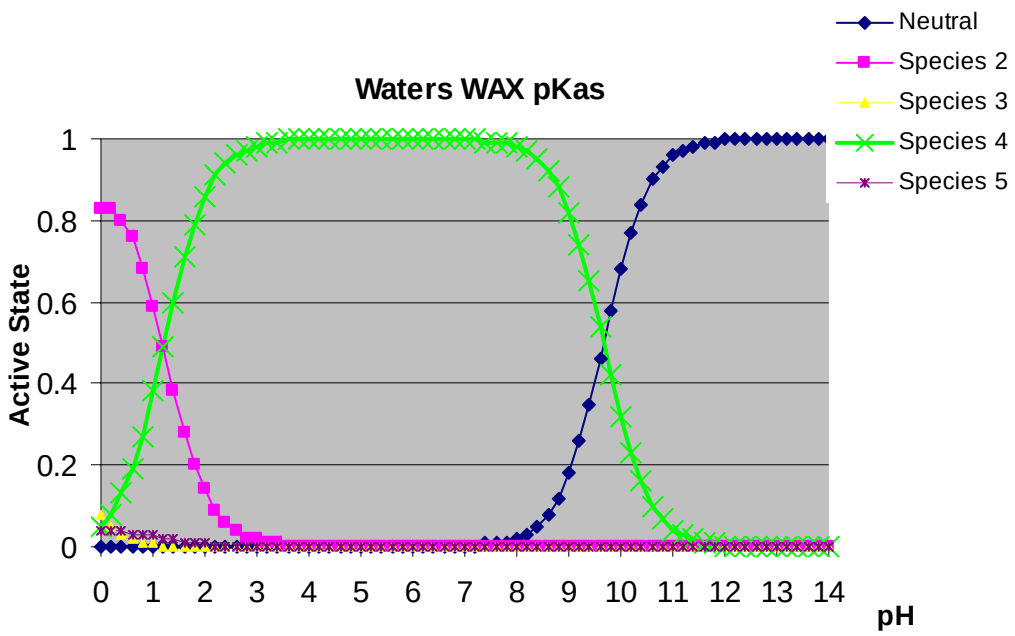


Figure 3.21 Waters OASIS Active State According to pH (color matches above structures).

As shown in both figures, both cartridges remain ionized approximately at pH<8.5. Above this pH, the active surface groups on the sorbent become neutralized and therefore unable to retain ionic compounds. Therefore, the working range for these cartridges to retain anionic analytes is with pH ranges <8.5. Most of the target analytes are ionic at this pH range with the exception of IMPY, TCPY, phorate sulfone and sulfoxide, terbufos sulfone and sulfoxide (Table 3.5) and therefore capable of utilizing the ionic exchange mechanism of these SPE cartridges. For the neutral analytes, the advantage of dual-retention capacity with the mixed mode cartridge will be investigated.

The general procedure for SPE weak-anion exchange is to activate the ionizable components in the SPE sorbent with a pH modified conditioning solvent (ex. 2% formic acid in methanol), followed by sample loading. After the sample is applied to the

cartridge, the cartridge is washed with a solvent(s) not compatible to the target analytes. For example, if isolating anionic compounds, the sorbent could be washed with 100% methanol to remove hydrophobic, neutral components extracted in the matrix. Hydrophobic interactions are weaker than ionic interactions (Table 3.8) therefore a wash step with high percentage of organic can aid in removing many matrix interferences without target analyte removal.

Interaction Type	Energy (kcal/mol)
Dispersion	(1-5)
Dipole-Induced Dipole	(2-7)
Dipole-Dipole	(5-10)
Hydrogen Bonding	(5-10)
Ionic	(50-200)
Covalent	(100-1000)

Table 3.8 Bond Energies *adapted from* (Hennion 1999).

The last step is elution with a solvent that will release target analytes from the sorbent bed. For weak anion-exchange mechanisms, this can be accomplished by either increasing the pH 2 units above the sorbent bed pKa (effectively neutralizing the bed) or by increasing the ionic strength of the elution solvent thereby promoting competition between counter-ions in the buffer with the target charged analytes (Varian 1993). For this analysis, altering the pH combined with an organic solvent to release ionic and neutral analytes will be the target action for elution because increasing the counter-ion strength alone will not aid in elution of neutral analytes. The volume of appropriate solvent to release target analytes from the sorbent bed is also parameter that can be investigated. However, the manufacturer recommended elution volume for a 200mg sorbent bed of 4mL will be used for SPE research (Phenomenex).

All of these steps require optimization for the target analytes to maximize valued method parameters like selectivity and sensitivity. For mixed-mode operations, the wash step is critical for analytes that are partially ionized and/or neutral at loading pH; recovery will decrease if the organic additive in the wash solvent is too high. Typically, the wash solvent entails high percentage organic to remove neutral analytes; however, several of the target analytes for this research are neutral at the loading pH and will have to be monitored closely for breakthrough at higher organic washes. Alternatively, fractionating heterogeneous mixtures of ionic and neutral analytes is possible with mixed mode applications for the same reason (Hennion 1999). Also, the advantage of mixed mode should be pronounced with ionized to partially ionized analytes that are capable of hydrophobic interactions (Table 3.5).

Degradation Product SPE Method Development

Target analytes were prepared in individual stock solutions; 2-5mg of native analyte was diluted with 15mL acetonitrile. The working standard stock solution was prepared by the addition of an aliquot of each individual stock solution to result in a concentration of 4 parts-per-million (ppm) (µg/mL). Likewise, individual stock internal standard solutions were prepared similarly with 2-5mg of label analyte diluted with 15mL acetonitrile. The working label standard stock solution was prepared from aliquots from individual stock solutions to give a resulting concentration of 10ppm For all initial SPE optimization experiments, spiked water standards (100ng/g) were used to focus on analyte-SPE sorbent retention mechanisms and behavior before matrix standards were introduced. In order to maximize retention on the ion exchange sorbents, solutions containing the target

analytes should be loaded at a pH where all the ionic groups (both analytes and sorbent) are charged. Therefore, loading solutions at pH 3-7 were investigated for maximum retention.

Counter-ion strength and selectivity are important factors of ionic exchange mechanisms. Ion exchange (IE) is a competitive mechanism and to ensure selective retention of target analytes over buffer counter-ions, buffer relative selectivity and concentration should be low. Table 3.9 below is a list of relative counter-ion strength of common ions found in buffers with highest counter-ion strengths reported with Ba^{+2} and Benzene Sulfonate.

CATIONS		ANIONS	
Li^{+1}, H^{+1}	0.5	OH^{-1}, F^{-1}, propionate	0.1
Na^{+1}	1.5	Acetate, Formate	0.2
$(NH_4)^{+1}$	2.0	$(HPO_4)^{-2}$, $(HCO)^{-1}$	0.4
Mn^{+2}, K^{+1}, Mg^{+2}, $Fe^{+2,+3}$	2.5	Cl^{-1}, $(NO_2)^{-1}$	1.0
Zn^{+2}, Co^{+2}, Cu^{+1}, Cd^{+2}	3.0	$(HSO_3)^{-1}$, CN^{-1}	1.5
Ca^{+2}	4.5	$(NO_3)^{-1}$	4.0
Cu^{+2}	6.0	$(ClO_3)^{-1}$	4.5
Pb^{+1}, Ag^{+1}	8.5	$(HSO_4)^{-1}$	5.0
Ba^{+2}	10.0	Citrate	9.5
		Benzene Sulfonate	10.0

Table 3.9 Relative Counter-Ion Strength for Common Buffers *Adapted from* (Snyder 1997).

Acetate and phosphate buffers were chosen to control pH of each experiment because of their desirable pKa (4.76 and 7.2 respectively) and low counter-ion selectivity (0.2 and 0.4 respectively) (Snyder 1997; Agilent 2006). Buffers were prepared (acetate or phosphate) at 0.1M to achieve the pH selected for consideration. A flowchart of the protocol followed for initial SPE investigation is shown below in Figure 3.22.

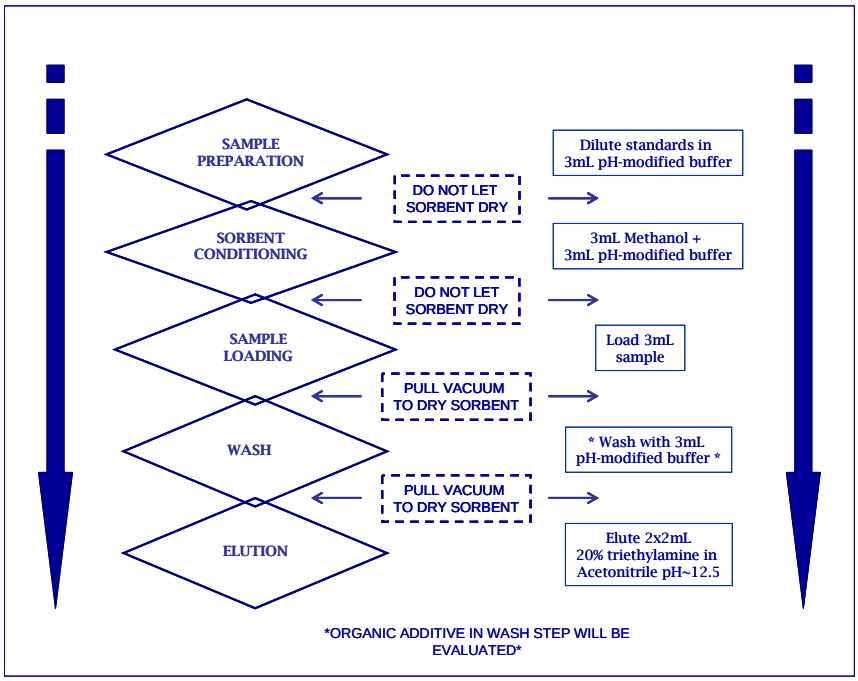


Figure 3.22 Flowchart of Ion Exchange SPE Method Development.

The following recoveries were obtained for both SPE products with different buffers at various pH/buffer conditions and are summarized in Table 3.10 below. All recoveries were normalized with "100%" recovery samples that were spiked after SPE.

	Buffer & SPE Cartridge Selection for Sample Preparation N=5									
	0.1M Acetate pH 3.0		0.1M Acetate pH 3.5		0.1M Acetate pH 4.0		0.1M Phosphate pH 5.7		0.1M Phosphate pH 7.0	
Analyte	Strata	Oasis	Strata	Oasis	Strata	Oasis	Strata	Oasis	Strata	Oasis
4-NP	94	91	91	90	96	94	93	77	87	105
IMPY	97	57	91	54	80	49	104	53	100	54
TCPY	92	91	88	85	99	93	91	98	89	88
MDA	37	42	28	31	61	79	92	111	84	97
DCCA	97	97	102	94	108	92	99	103	102	102
DBCA	84	91	89	84	103	83	86	109	77	102
CDCA	40	21	31	22	74	74	81	93	85	120
CFCA	86	82	85	84	74	85	107	131	106	105
3PBA	89	84	86	91	89	91	88	97	97	100
4F3PBA	98	84	82	81	100	103	79	113	99	98
Phorate Sulfone	95	86	91	93	90	71	82	65	76	52
Phorate Sulfoxide	98	74	94	71	91	70	84	77	77	85
Terbufos Sulfone	90	79	94	65	120	64	105	71	106	55
Terbufos Sulfoxide	88	89	91	84	79	114	84	81	118	79
DMP	96	46	92	54	99	21	15	9	15	2
DEP	92	85	95	87	96	82	55	26	36	14
DMTP	88	79	92	91	101	89	99	93	47	58
DETP	94	91	91	76	101	93	83	99	95	89
DMDTP	99	98	93	87	88	93	86	94	101	96
DEDTP	95	84	89	88	98	102	91	93	102	99

Table 3.10 Percent Recovery for Target Analytes under Various Conditions Comparing Strata and OASIS SPE.

Most of the target analytes showed comparable results for both cartridges. However, IMPY exhibited dramatic differences between the two cartridges at all conditions. Presumably, IMPY is retaining to the sorbents with hydrophobic interactions because it is in the neutral state at each pH investigated with a high log D value. The OASIS sorbent bed contains many aromatic structures thus increasing hydrophobicity overall (Π-Π interactions) (see OASIS sorbent bed structures above). Perhaps IMPY has a stronger retention with the OASIS cartridge and although the elution solvent is highly organic (20% triethylamine in acetonitrile), it may not be strong enough to release the IPMY analyte from the OASIS sorbent. This conclusion however, contradicts the high recoveries seen with both 4-nitrophenol and TCPY which are both neutral at the

investigated pH and are both nitrogen-containing aromatic structures. The low log D value (~-0.44) may help explain a lower partitioning with the hydrophobic moieties for 4-nitrophenol.

Results indicate a direct correlation between pH and DMP/DEP retention with both cartridges, although recoveries for the Strata cartridge are more pronounced between pH 4 and pH 5. It is not clear however, if this is a result of the change in pH or possibly the change in buffer. Both analytes are in the ionized state at each investigated pH (excluding pH3 where they are both partially ionized). Therefore, the more likely conclusion is that the change in buffer has a higher impact on retention of DMP/DEP. As stated previously, buffer counter-ion selectivity is a concern when contemplating ion-exchange mechanisms. Although both phosphate (0.4) and acetate (0.2) anions exhibit low affinity for ion-exchange sorbents, perhaps the 0.2 difference between the two is relevant to retention of DMP/DEP and competition with counter-ions and the active sites (Agilent 2006). Perhaps this difference is enhanced by the higher buffer concentration (0.1M) utilized for the procedure, a concentration that by some standards may be too high for ion exchange mechanisms (Weber 2001).

Inversely, MDA and CDCA exhibited almost the exact opposite behavior as that of DMP/DEP. Both MDA and CDCA had poorer recoveries at lower pH (3-3.5) and/or the acetate buffer used. However because both analytes showed better recoveries at pH4 using the acetate buffer, it is assumed that pH is more influential in retention than buffer selection or concentration. Both of these analytes are partially ionized at lower pH which

may affect their retention behavior as opposed to a higher pH where they predominantly exist in their charged state (>pH 4 for MDA and >pH5 for CDCA). Despite several other target analytes that have similar pKas and ionizable behaviors, the log D value for MDA and CDCA are lower. This lower log D value could indicate that these analytes at lower pH are more hydrophilic and less likely to interact with hydrophobic regions of the sorbent bed.

Because MDA/CDCA and DMP/DEP have contrasting behaviors, achieving high recoveries collectively is difficult. However, all analytes have recoveries >70%, at pH4 using the 0.1M acetate buffer with the Strata SPE cartridge, except MDA (61%). Therefore, the method with 0.1M acetate buffer at pH4 using the Strata SPE cartridges was chosen for further SPE development.

The next step for investigation is the wash step of the SPE protocol. Analysis of this step will elucidate specifically hydrophobic interactions of the target analytes with the SPE sorbent at pH 4. Similar to the wash step analysis of the parent pesticide analytes with SPE, varying organic percentages for the wash solvent will be used to determine analyte break-thru. The only difference between the two assays is that this procedure will involve wash solvents with varying organic percentages at pH 4. Figure 3.23 below depicts the flow of steps used in the wash step analysis.

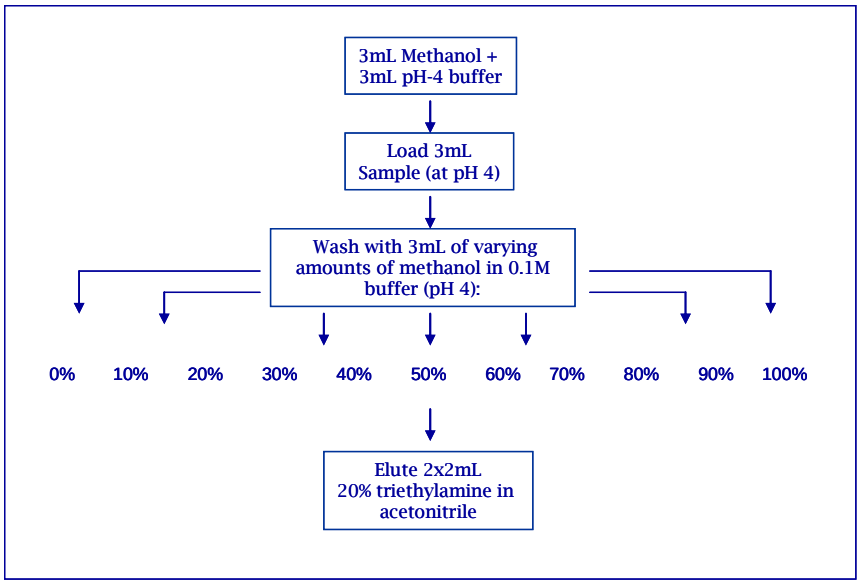


Figure 3.23 Flowchart of Wash Analysis for Ion Exchange SPE.

The purpose of the wash analysis with varying percentages of organic modifier added to the buffer is to observe the point at which all analytes break-thru the SPE sorbent. Additionally, this analysis will further clarify if each analyte is retaining to the sorbent through hydrophobic interactions. The following recoveries were obtained normalized to "100%" recovery samples spiked after SPE and are summarized below in Table 3.11.

	Wash Step Analysis with Varying Percentage Methanol Additive in 0.1M Acetate Buffer (pH 4) N=5																			
% Organic in Aqueous Buffer	4-NP	DBCA	CFCA	DCCA	TCPY	3-PBA	4F3-PBA	CDCA	MDA	IMPY	Terbufos Sulfone	Phorate Sulfone	Terbufos Sulfoxide	Phorate Sulfoxide	DMP	DEP	DMTP	DETP	DMDTP	DEDTP
0	95	81	83	102	91	100	100	63	69	96	90	102	88	99	95	94	99	84	93	84
10	92	93	81	86	85	84	80	67	68	102	86	100	90	91	81	86	87	82	83	82
20	98	90	85	98	109	91	92	64	61	82	100	101	84	100	91	94	94	91	91	89
30	102	100	88	100	100	90	91	43	44	80	101	107	86	102	98	100	90	97	95	90
40	91	94	85	91	80	89	95	40	43	12	108	100	87	101	94	93	94	97	87	83
50	100	86	89	92	93	94	95	40	9	6	96	102	85	108	98	102	96	102	82	93
60	85	81	76	87	84	81	87	9	7	4	64	56	70	77	88	83	94	80	96	77
70	96	96	78	99	85	86	93	5	2	3	70	76	71	75	101	88	99	92	89	84
80	90	98	71	82	90	87	95	6	0	3	79	71	48	14	93	82	84	97	89	83
90	14	92	0	37	93	80	86	0	0	2	13	10	6	5	97	96	91	90	97	85
100	7	94	0	33	92	86	92	0	0	0	9	7	6	4	99	89	99	97	89	90

Table 3.11 Percent Recovery for Target Analytes under Varying Wash Conditions.

A graphical example of three analytes and their retention behavior with varying organic washes is shown in Figure 3.24 below.

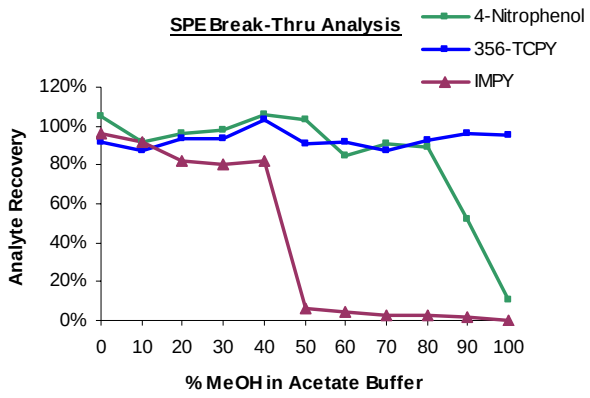


Figure 3.24 Selected Analyte Break-thru Analysis for the Wash Step.

Many of the target analytes followed predictable retention behavior as specified by their charge state and log D value. The pH-independent neutral analytes (the sulfones and sulfoxides) all exhibited a similar break-thru pattern starting approximately at 60% methanol. This indicates that at least 60% organic solvent is necessary to break hydrophobic interactions between these analytes and the sorbent bed. As shown above 4-nitrophenol, which is neutral at pH 4, began to break-thru after 80% methanol was applied. IMPY also demonstrated break-thru but with less organic at 40% methanol. Both IMPY and 4-nitrophenol corroborate with their prior analyses- that both are existing primarily in their neutral state at pH 4. The fact that 4-nitrophenol has a much lower log D value (-0.44) than that of IMPY (2.84) also supports these results because IMPY has a greater tendency to partition with organic solvents which is shown with its break-thru pattern at a much lower % methanol.

Surprisingly, 356-TCPY does not behave as predicted by any calculated values. Not only is it primarily in the neutral state at pH 4, but it also has the highest log D value of all the target analytes and it is an extremely weak acid (pKa ~10). From the break-thru analysis, it appears that 356-TCPY does not break-thru with any % methanol suggesting that other mechanisms are promoting its retention to the sorbent bed.

Again, MDA and CDCA show similar behavior patterns; both have significant break-thru after approximately 50% methanol which leads to the conclusion from the earlier analysis that both analytes probably exist as both neutral and charged entities at pH 4 and can therefore have both ion exchange and hydrophobic tendencies toward the sorbent bed. All of the DAPs do not break-thru at any % methanol confirming calculated values that they exist primarily in the charged state at pH 4. The 30% methanol in aqueous buffer was chosen as the wash solvent for further analysis largely due to the loss of IMPY at higher organic washes. Although MDA and CDCA have lower recoveries at this wash, their overall recoveries are lower due to the pH chosen for further evaluation.

Degradation Product Initial Soil Extraction

In order to extract degradation products from soil with SPE, the soil must be converted to the liquid phase. Two different centrifugal filter devices were considered for initial soil extraction: Whatman VectaSpin 20™ polypropylene mesh (10µm) (Brentwood, UK) and Millipore Amicon® Ultra-15 cellulose membrane (5000 nominal molecular weight limit) (Billerica, MA) to observe extraction recoveries between different filter mechanisms. Initial extraction assays were first performed with 1 (3mL buffered water) extraction to

focus on the differences between filter devices as well as initial extraction pH. Ionizing target analytes should promote extraction with buffered water solvents thus pH is an important parameter to consider for this step. At pH 7, all target analytes are primarily in their charged state (except IMPY, TCPY, sulfones, sulfoxides and 4-NP is partially ionized) which would improve extraction recoveries with water. Additionally, it has been shown that extraction with a higher pH reduced the amount of humic acid co-extracted with the target analytes (Pichon, Coumes et al. 1996; Niessen, Manini et al. 2006). This has been shown to created problems for methods with non-MS detectors that result with large solvent fronting in the chromatogram. Humic acid tends to exaggerate this peak often interfering with target analyte chromatography. Although this method will utilize MS instrumentation, eliminating the solvent front, reducing this possible matrix effect is beneficial to the analysis. Initial extraction at pH 4 was also analyzed so that an acidification step did not need to be performed prior to SPE (to bring pH 7 samples to pH 4 for SPE loading). The following flowchart shown in Figure 3.25 below shows the steps taken for the extraction step for method development. The following Figure 3.26 shows recoveries for the two-dimensional analysis comparing both filter devices at pH 4 and pH 7.

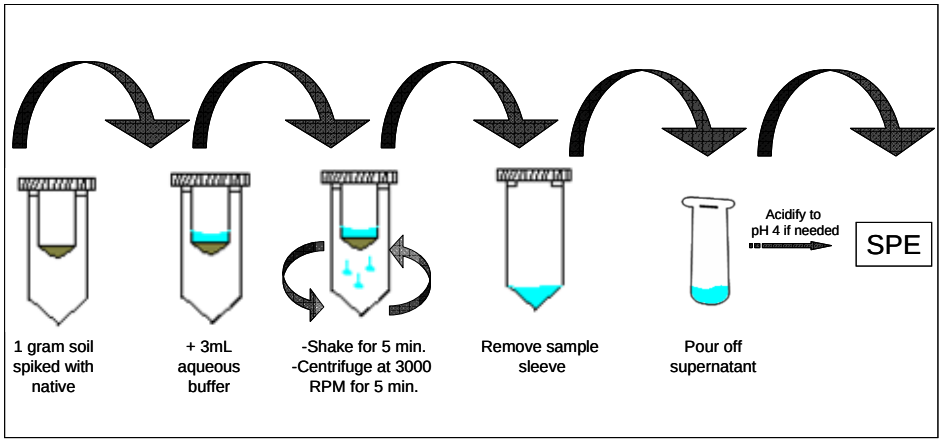


Figure 3.25 Initial Soil Extraction Protocol.

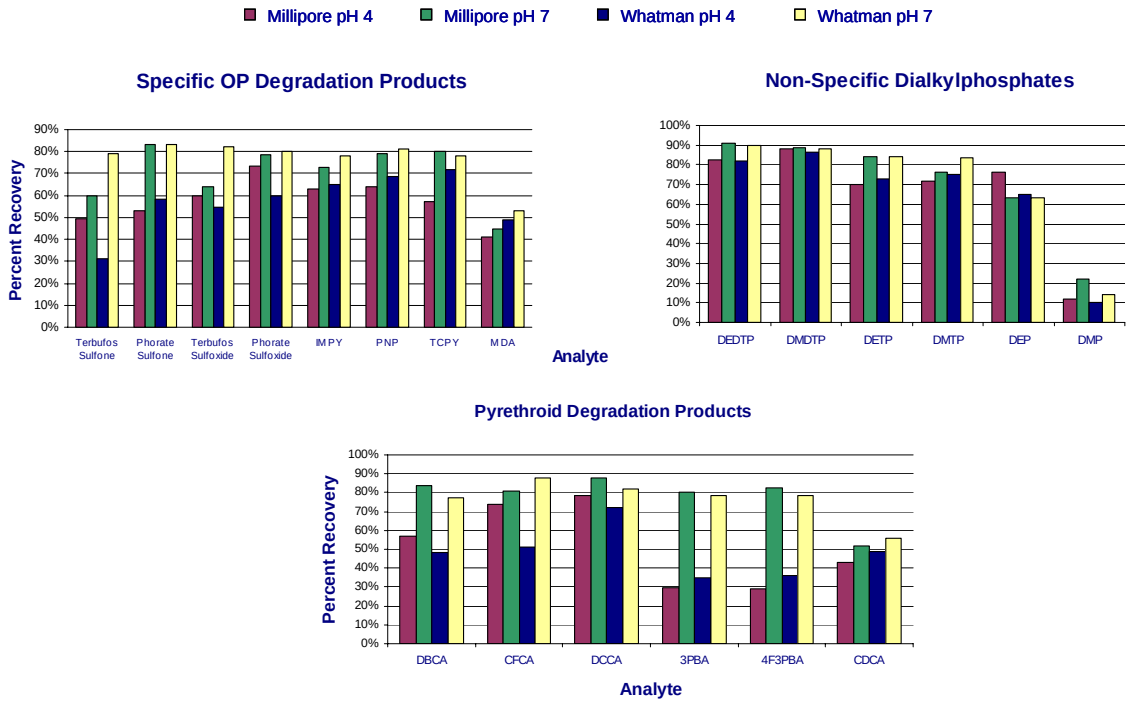


Figure 3.26 Percent Recovery for Target Analytes Comparing 2 Filter Devices at pH4 and pH7.

Collectively, the filter device did not affect recoveries as significantly as the extraction pH. The trend for the specific OP and pyrethroid degradation products shows higher recoveries for both filter devices at pH 7 compared to pH 4, although recoveries for the Whatman filters were slightly higher than the Millipore filters. Interestingly, this trend was not observed for the DAPs where there was little difference between conditions investigated. DMP showed an extreme loss of recovery utilizing all filters at both pH conditions with less than 20% recovered. As stated before, DMP (and DEP) is sensitive to pH change. Theoretically, DMP should be more ionizable and therefore more "extractable" under aqueous conditions at high pH. The pKa for DMP is 2 and at pH 7, the log D value is -2.44. Under these conditions with these calculated values, DMP should have higher recoveries with conditions progressively higher in pH because it is

ionized and partitions with aqueous phases. However, the experimental data does not match theoretical data for DMP. Other than DMP, most of the target analytes maintained the high recoveries (for the Whatman filter device with conditions at pH 7) seen without matrix. The analytes that had recovery loss include: terbufos sulfone (70%), 4-NP (80%), TCPY (80%) and DEP (60%) albeit high recoveries overall. To assess whether additional extractions could improve recoveries for the target analytes, a multiple extraction assay was investigated. Figure 3.27 below describe the behavior of target analytes with increased number of extractions.

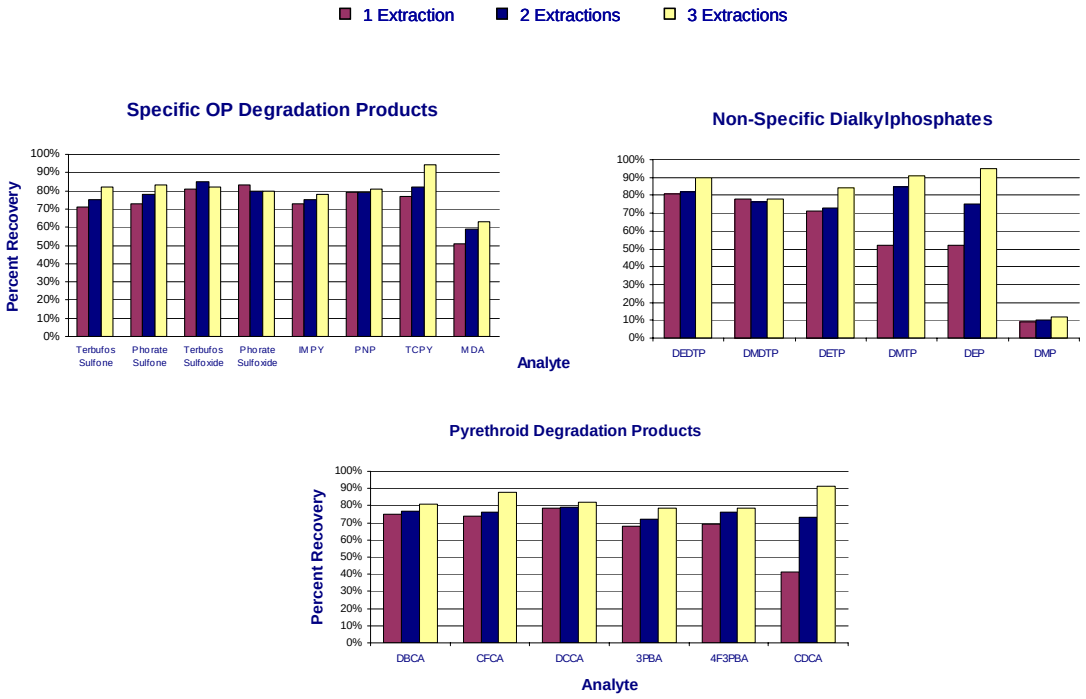


Figure 3.27 Percent Recovery for Target Analytes with Multiple Extractions at pH7 with the Whatman Filter Devices.

Surprisingly, few of the target analytes showed any improvement with increased number of extractions. Marked improvements were shown with DMTP, DEP and CDCA where

percentage recoveries doubled. Overall, the highest recoveries were with three extractions and thus, three extractions will be used for overall method validation.

Method Validation

The conventional analytical parameters needed to validate this method were evaluated and then discussed in detail. Additionally, matrix effects were investigated thoroughly to determine if the matrix of system components affect the investigated method validation parameters. For example, the percentage recovered from a sample may be affected by matrix effects, thus giving a value that is a result of the overall method efficiency but not the true analyte recovery. This differentiation is important, however, hardly ever investigated or mentioned in analytical publications (Niessen, Manini et al. 2006).

All concentrations for quantitation purposes are defined in Table 3.12 below. These materials will be used for all subsequent evaluations.

Standard Curve and Quality Control Concentration Summary *(ng/g)*

	Standard Curve							Quality Control	
Analyte	S7	S6	S5	S4	S3	S2	S1	Low	High
3PBA	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
4F3PBA	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
CFCA	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
DCCA	1454.5	727.3	363.6	181.8	90.9	45.5	22.7	150.0	275.0
IMPY	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
MDA	363.6	181.8	90.9	45.5	22.7	11.4	5.7	25.0	100.0
Terbufos Sulfone	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
Terbufos Sulfoxide	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
Phorate Sulfone	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
Phorate Sulfoxide	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
4- Nitrophenol	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
356 TCPY	363.6	181.8	90.9	45.5	22.7	11.4	5.7	25.0	100.0
DBCA	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
DMP	2909.1	1454.5	727.3	363.6	181.8	90.9	45.5	350.0	550.0
DMTP	363.6	181.8	90.9	45.5	22.7	11.4	5.7	25.0	100.0
DMDTP	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
DEP	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
DETP	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
DEDTP	181.8	90.9	45.5	22.7	11.4	5.7	2.8	25.0	100.0
CDCA	800.0	400.0	200.0	100.0	50.0	25.0	12.5	25.0	100.0

Table 3.12 Summary of Standard Curve and Quality Control Spiked Concentrations.

Detection Limits

The limits of detection for the degradation products were determined with a slightly different method than was calculated with the parent pesticides. In both cases, the Taylor method was used to determine both detection limits and quantitation limits of the method, however, there is a slight variation with the calculations for the degradation product detection limits (Taylor 1987). 14 standard curves were prepared (S1-S7) and analyzed using 7 of the standard curves as the "standard curve" and the other 7 curves as "unknowns". Each set (1 standard curve versus 1 unknown standard curve) was run for 7 consecutive days. Instead of graphing the standard deviation at each level against the actual standard concentration to find the *y intercept*, the standard deviation of the actual calculated values (as determined from the standard curve) are plotted against the actual

standard concentration. Both detection limit calculations are accepted forms of calculating detection limits. However, the advantage of the second calculation method is that it is a more specific measurement for the selected analytical method. It takes into account variation, over time, associated with standard curves, instrumentation, sample preparation technique, etc. that is typical to any analytical method. It also defines the correlation between spiked amounts and the actual calculated values in association with the standard curve. As a result, it is a better indicator of how the method will behave with "real" samples. As with the parent pesticide analytes, the detection limit is calculated as 3* S_0. Figure 3.28 below shows the *y intercept* of 4-NP.

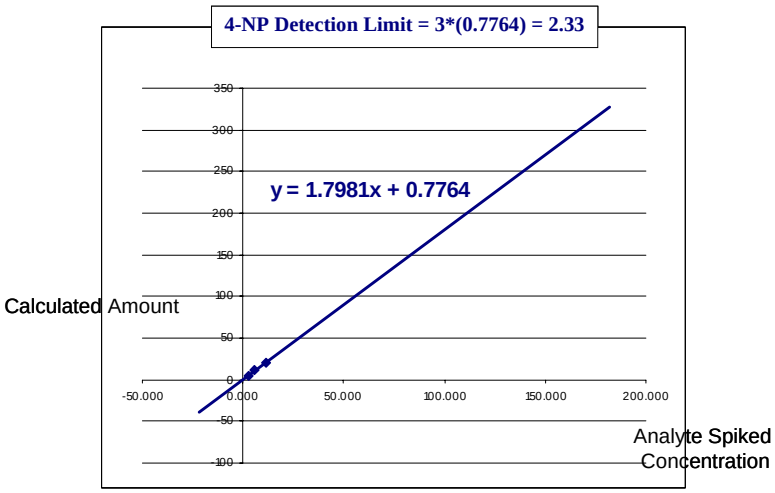


Figure 3.28 Determination of LOD for 4-Nitrophenol with Taylor Method.

Table 3.13 below gives all of the detection limits (ng/g) for the degradation products as well as the limit of quantification (10*S_0), which defines the concentration that the method can quantitate at higher certainty that the LOD. (Taylor 1987).

	y intercept	**LOD**	**LOQ**
4-NP	0.78	2.33	7.76
DEDTP	0.25	0.75	2.49
DBCA	1.67	5.00	16.66
CFCA	1.66	4.97	16.56
DCCA	9.63	28.90	96.34
DMDTP	0.67	2.01	6.70
TCPY	3.44	10.32	34.39
3PBA	0.11	0.33	1.11
4F3PBA	0.20	0.59	1.98
DETP	1.41	4.22	14.08
DMTP	0.15	0.45	1.49
DEP	0.13	0.39	1.29
CDCA	6.29	18.87	62.89
MDA	2.81	8.42	28.08
DMP	28.56	85.67	285.56
Terbufos Sulfone	1.11	3.34	11.13
Phorate Sulfone	1.30	3.90	12.99
Terbufos Sulfoxide	2.23	6.70	22.35
Phorate Sulfoxide	0.88	2.65	8.84
IMPY	0.04	0.13	0.42

Table 3.13 Limit of Detection and Quantitation for Target Analytes.

The range of detection limits are between the low ppt to the low ppb range (.13-85.67 ppb). The higher detection limits (DMP, CDCA, TCPY, DCCA) are most likely due to higher variability in the analyses that affect the correlation between calculated and spiked concentration values. These analytes may be more sensitive to matrix effects, a topic of discussion later. DMP specifically is problematic overall with this method as will be obvious in other validation discussions. Most likely, the lack of recovery for DMP contributes to the lower sensitivity and therefore precision and accuracy of DMP using this method.

As shown with the parent pesticides, the Taylor method generally gives conservative values for detection limits. The chromatograms in Figure 3.29 below show the signal to noise ratio (SN) for the listed target analytes at 2.84ng/g (in matrix). DMP is shown at

727.27ng/g (in matrix). Although the calculated values for the sulfones and the sulfoxides give detection limits between 2.65-6.70ng/g, high SN ratios clearly indicate that the detection limits are much lower for these analytes. IMPY, which gives a calculated LOD value of 0.13 is confirmed with the high SN ratio at 2.84ng/g. In contrast, DMP which has a calculated detection limit value of 85.67 is underestimated with the Taylor method; the SN ratio at 363.6ng/g is 39 (SN ratio at S4 concentration of 181.8ng/g is inconsistently between 0-10) which clearly indicates a higher detection limit for DMP than given.

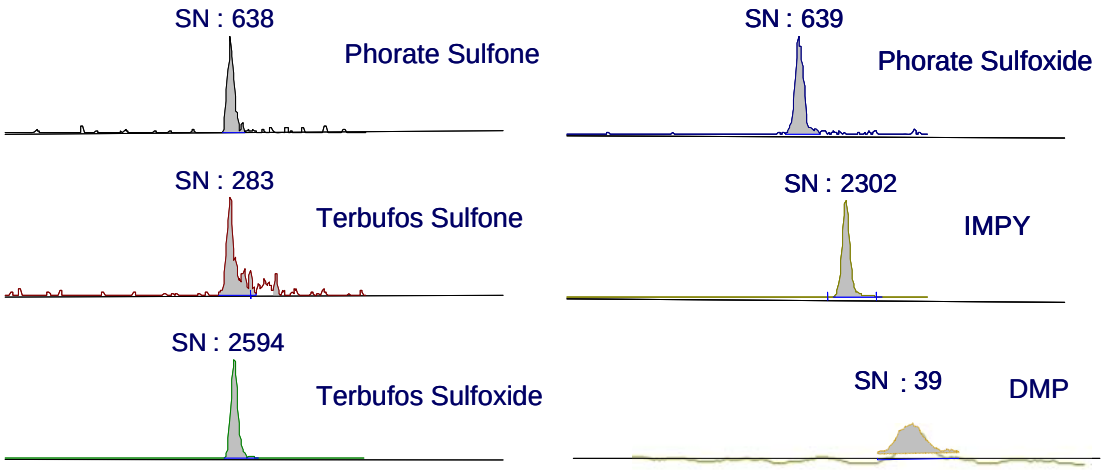


Figure 3.29 SN Ratio for Selected Analytes at 2.84ng/g (DMP 727.27ng/g).

Analyte Recoveries

Analyte recoveries were determined at S4 and S7 for each analyte by spiking thirty samples:

1. Prior to all extraction and instrumentation (10 samples),
2. After soil extraction but before SPE extraction (10 samples) and
3. After all extractions bur before instrumentation (10 samples)

The group 3 spikes were considered the "100" recovery samples. Groups "1" and "2" were normalized to group "3" spikes to determine the amount of analyte loss at each extraction step. Overall recoveries are shown in Figure 3.30 and Figure 3.31 below grouped by MS scan polarity.

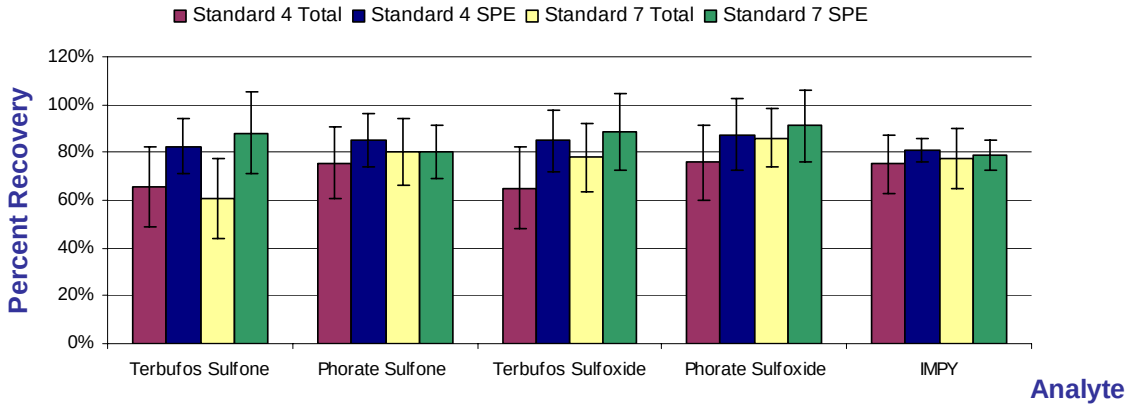


Figure 3.30 Percent Recovery for Target Analytes Scanned in Positive Mode at S4 and S7.

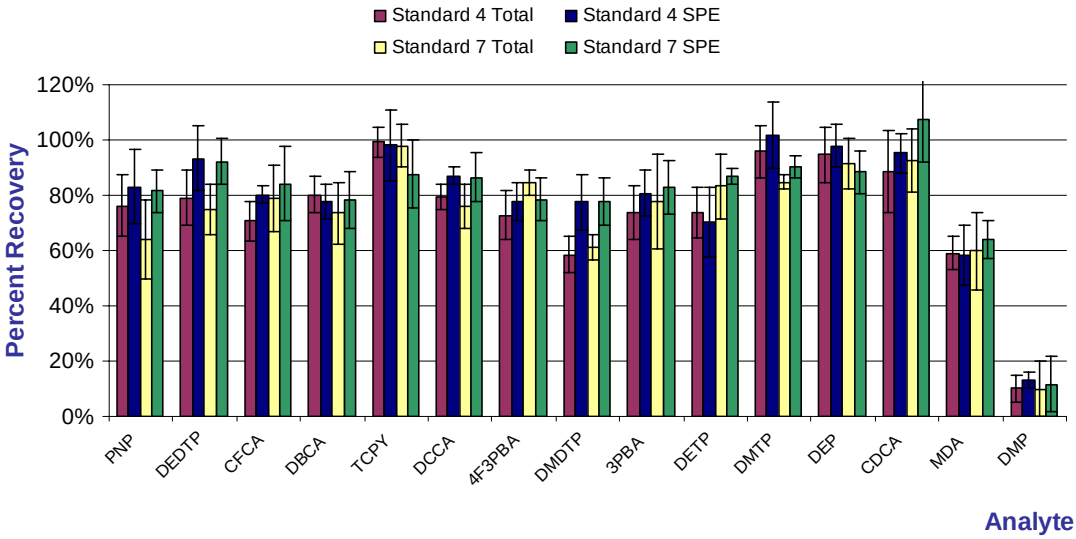


Figure 3.31 Percent Recovery for Target Analytes Scanned in Negative Mode at S4 and S7.

The recovery samples generally agree with the previous recovery investigations. Between 10-20% of target analyte was lost during initial soil extraction (prior to SPE) for

the sulfones and sulfoxides. This is most likely due to the neutral state and/or high log D values of these analytes and tendency to partition to organic and not aqueous phases. There is a notable recovery loss (~20%) between initial extraction and SPE for DMDTP and DEDTP as well which is inexplicable. The loss could be a factor of the higher log D values for these two analytes (in comparison with the other DAPs). However, this conclusion is contradictory to the wash analysis results. If DMDTP and DEDTP were behaving as "neutrals", an increasing loss of analyte should be observed with higher organic washes. Although the two extractions (initial soil extraction and SPE) are performed under two different pHs (pH 7 for initial extraction and pH 4 for SPE), there is only a negligible difference between log D values and thus should have no affect on analyte behavior thereof. There was very little difference in recoveries for the two concentrations showing agreement. Overall total recoveries were between 59-99% for S4 and 60-98% for S7 (excluding DMP which gave recoveries of 10% for both S4 and S7).

Quality Control Characterization

Quality control materials were prepared by spiking specific analyte concentrations (see Table 3.12) into 50grams of blank soil for QC-low (QCL) and QC-high (QCH) pools. A third QC pool was not spiked and after assurance that this QC pool contained no endogenous target analytes, it was used for blanks and standard curve materials for QC and future unknown sample analysis. QC pools were diluted with 100mL acetonitrile, covered, shaken and stirred for 48 hours after which tops were removed and both pools allowed to air dry for another 48 hours. QC pools were then placed in -20ºC freezer to prevent further degradation of target analytes. Each day for 6 days in succession, 6

discrete aliquots of 1g of QCL and 1g of QCH were analyzed against 1 blank and 1 standard curve (S1-S7). Results from the 6 days were used to calculate accuracy and precision parameters to describe the overall method. A summary of calculated values are shown in Table 3.14.

Analyte	Accuracy		Precision (RSD)					
	Average (%dev)		Within Day		Between Day		Overall	
	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*
PNP	26.3 (5.2)	90.1 (-8.9)	10.0	7.8	20.8	16.5	15.3	14.0
DEDTP	12.2 (-51.2)	34.2 (-65.8)	12.0	17.8	27.7	25.9	15.9	14.3
DBCA	24.6 (-1.6)	93.1 (-6.9)	18.0	5.1	23.0	19.1	24.7	12.5
CFCA	27.1 (8.4)	104.1 (4.1)	31.8	21.2	11.0	21.3	51.9	36.1
DCCA	148.9 (-0.7)	264.8 (-3.8)	9.0	5.6	8.6	9.8	9.2	8.3
DMDTP	3.15 (-87.2)	14.4 (-85.6)	13.0	10.9	36.1	27.7	23.5	14.7
TCPY	25.5 (2)	103.2 (3.2)	19.0	6.4	16.4	21.1	17.6	15.7
3PBA	23.4 (-6.4)	90 (-10)	8.2	4.6	9.2	6.3	10.0	8.3
4F3PBA	20.1 (-19.6)	84.8 (-15.2)	10.4	7.8	7.9	9.1	12.7	7.4
DETP	12.1 (-52)	47.9 (-52.1)	9.4	8.5	14.9	13.7	13.0	10.9
DMTP	21.09 (-15.6)	78.3 (-21.7)	12.7	8.4	22.0	14.5	14.7	10.5
DEP	27.1 (8.4)	110.1 (10.1)	8.5	5.8	12.5	18.1	10.8	9.3
CDCA	14.8 (-40.8)	94.9 (-5.1)	11.0	7.4	24.8	14.5	31.1	12.1
MDA	24.2 (-3.2)	98.2 (-1.8)	11.1	7.7	9.6	6.8	16.4	8.7
DMP	246.6 (-1.4)	436.8 (-2.9)	4.8	3.6	12.8	10.9	11.8	8.8
Terbufos Sulfone	21.6 (-13.6)	86.7 (-13.3)	14.0	6.6	38.0	11.5	34.0	15.1
Phorate Sulfone	19.7 (-21.2)	78.9 (-21.1)	13.6	6.2	33.4	14.7	33.5	15.2
Terbufos Sulfoxide	30.6 (22.4)	91.5 (-8.5)	12.0	6.8	33.4	25.2	29.4	15.6
Phorate Sulfoxide	27.2 (8.8)	104.7 (4.7)	22.8	9.5	25.0	10.4	55.8	13.6
IMPY	25.7 (2.8)	108.1 (8.1)	15.1	6.3	22.5	13.1	17.1	9.4

Table 3.14 Accuracy and Precision Summary for Target Analytes in Soil.

Precision was determined by calculating relative standard deviation of the repeat analyses of quality control materials (*n=36* for QCL and QCH). Within-day precision describes the variation of calculated values within the same batch on the same day whereas between-day precision accounts for the variation between the 6 days of analysis. Overall RSD was calculated for all target analytes as the average relative standard deviation for all samples at all days. The average within-day, between-day, and total RSD was 8.2%, 15.5% and 13% respectively for all target analytes at QCH. Within-day variation was

higher at lower concentrations (QCL – 13.3%). All target analytes had overall RSDS ≤ 16% (QCH) except for CFCA which had high RSD (36.1%).

Figure 3.32 below shows the difference between high variation (CFCA) and low, acceptable variation (3-PBA). Higher variation seen at lower concentrations gave less target analytes with RSDs ≤ 16%. Only 9/20 analytes has overall RSD values ≤ 16% at the QCL concentrations. This indicates that this method should only be used for qualitative purposes at the lower end of the standard curve as variation is too high for precise quantitation. This is most likely due to high RSDs for between-day precision seen with most of the analytes. A similar trend was seen with the parent pesticides as well which may indicate an overall inadequate fortification scheme for QC pools.

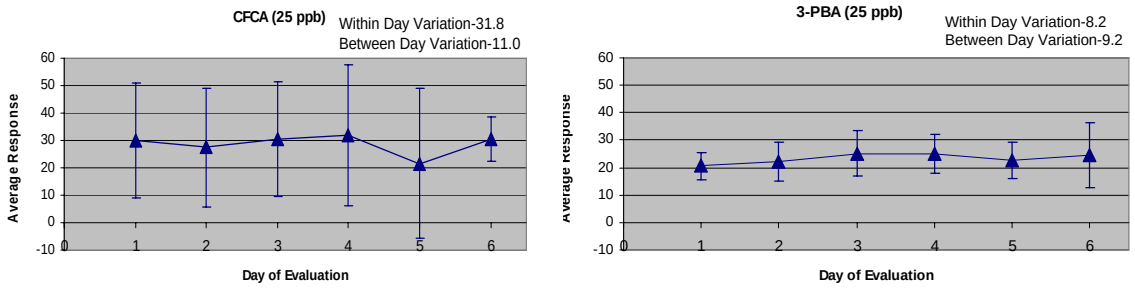


Figure 3.32 Variation over 6 Days for CFCA and 3-PBA.

Accuracy was calculated as the mean percentage deviation from the spiked value. For 8/20 target analytes, values exceeded 15% of the nominal spiked value (DMDTP, DEDTP, 4F-3PBA, DETP, DMTP, CDCA, Phorate Sulfone, Terbufos Sulfoxide). These analytes that showed high deviation were all less than the spiked value. This is most likely an indication of a poor soil fortification method, otherwise similar reductions in analyte recoveries would have mirrored such analyte loss from the spiked values.

Analyzing the accessibility of target analytes in soil over time to determine this discrepancy should be investigated further to determine where loss of analyte is occurring. It has been shown that for some analytes, tighter bonds are formed over time that can hinder extraction of analyte from soil matrices (Gevao, Semple et al. 2000).

Linearity

Although many of the analytes showed poor accuracy results from the QC characterization, all target analytes showed excellent correlation to spiked standard curve values. A summary of regression correlation coefficients of variation that were calculated using linear fits is shown in Table 3.15.

Linearity	
Analyte	R^2
PNP	0.989
DEDTP	0.995
DBCA	0.995
CFCA	0.994
DCCA	0.991
DMDTP	0.995
TCPY	0.996
3PBA	0.994
4F3PBA	0.999
DETP	0.998
DMTP	0.987
CDCA	0.993
DEP	0.974
MDA	0.990
DMP	0.997
Terbufos Sulfone	0.991
Phorate Sulfone	0.993
Terbufos Sulfoxide	0.987
Phorate Sulfoxide	0.991
IMPY	0.996

Table 3.15 Correlation Coefficients of Target Analytes.

An example showing 4F-3PBA linearity with a correlation coefficient of 0.9987 is shown in Figure 3.33 below to demonstrate the high correlation between spiked standard amount and calculated value.

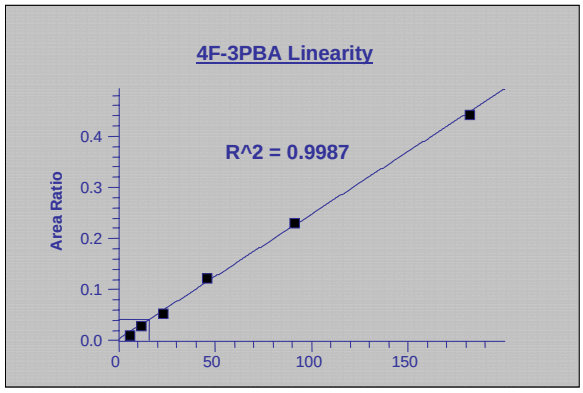


Figure 3.33 Linearity of 4F-3PBA.

Matrix Effects

As stated before, matrix effects can dramatically decrease the integrity of an analytical method. Non-ionized, co-extracted, co-eluted compounds such as salts, compounds with high surface activities and/or ion pairing properties, compounds with high proton affinities or low gas-phase acidities are typically the interferences responsible for deteriorated method validation parameters (Niessen, Manini et al. 2006). There are several ways to minimize introducing matrix effects into a method. Simply reducing the sample injection volume or diluting the sample can decrease the amount of unwanted compounds that are present in the ionization source. Unfortunately, this can also decrease the amount of target analytes as well so may not be an option for instruments with lower sensitivity. Another straightforward option is changing the ionization source, although ionization efficiency is analyte specific and if a large number of target analytes are involved, this solution may not be practical. Replacing surrogate or analog standards

with label internal standards will help to mask the effects of matrix related problems; label standards are often thought to correct the problem itself but this is actually incorrect and will be discussed later. The downside of utilizing label internal standards is that they are not always available for many analytes and/or they can be extremely expensive (Baker, Olsson et al. 2005; Hernandez, Sancho et al. 2005; Niessen, Manini et al. 2006). Probably the most laborious method for rectifying matrix effects is to improve the sample preparation method and/or change the mobile phase composition which requires revaluation of previous method development steps.

Mobile Phase Composition

There were several matrix effect-related problems encountered with this method and most were corrected after careful investigation. The first problem was due to the mobile phase composition. At the beginning of method development, the mobile phase determined to be optimal in terms of successful retention and complete separation with the HILIC column was the buffer which consisted of 95% acetonitrile in 5% 5mM ammonium formate buffer. This mobile phase was highly successful with solvent standard samples; no retention time shifting, normal peak shapes, very low maintenance over time. However, when real matrix samples were introduced to the instrument system, the LC column began clogging to a point where extreme pressures occurred that would eventually shut off the LC pump. To adjust for this problem, samples were pre-filtered before injection beginning with a 5µm Whatman filter, then switching to 2µm and then 1µm filter because the larger filters only prolonged the clogging. Although the 1µm filter corrected the clogging problem, it became apparent that approximately 50% of recovery

was lost for all of the analytes with the added filter step; DMP and DEP both had 0% recovery. The poor recoveries evident with the added filter step were unacceptable and warranted further re-evaluation of the problem.

The awareness of salts inherent in the soil matrix that are co-extracted with the target analytes can interfere with the ionization step was well known. However, there are no accounts of soil matrix components having such a profound effect on analysis *pre-ionization.* Salts are insoluble in organic solvents; however, with the 5% aqueous component of the mobile phase, the ammonium formate dissolved adequately and no problems were encountered with solvent standard evaluation. Perhaps the salt concentration, as a result of the addition of sample to the mobile phase, saturated the mobile phase causing precipitation to form in the LC column. This hypothesis would explain the clogging experienced with real samples versus not clogging seen with solvent standard samples. Simple experimentation confirmed the initial hypothesis: 1µL aliquots of extracted soil sample were added to 1mL of 6 different mobile phases with varying composition (listed below) to mimic mobile phase constitution.

1. 95% Acetonitrile -5% AF water
2. 95% Acetonitrile -5% AA water
3. 92.5% Acetonitrile – 7.5% AF water
4. 92.5% Acetonitrile – 7.5% AA water
5. 90% Acetonitrile – 10% AF water
6. 90% Acetonitrile – 10% AA water

AF = Ammonium Formate
AA = Ammonium Acetate

As predicted, addition of the sample to #1 resulted in an immediate cloudy solution. After ~1hour, a small amount of sediment appeared at the bottom of the solution. Decreasing the amount of organic to 92.5 % appeared to reduce initial cloudiness and the amount of sediment formed. At 90% organic, no cloudiness occurred and after ~24

hours, no formation of sediment appeared. The same trend occurred with ammonium acetate as the modifier; however, there was minimal amount of sedimentation present after ~24 hours and thus, method validation continued using the buffer with 90% acetonitrile in 10% AF water. An alternate solution could be to remove the amount of buffer in the mobile phase; however, as shown in the LC section of this research, buffer is needed for adequate retention with HILIC columns.

Lowering the organic portion of the mobile phase to 90% was the most reasonable solution to counteract precipitation. In addition to removing precipitation, another advantage with using lower organic is that run-time is shortened almost 50% (35min-20min). The consequence of using a higher organic/lower aqueous mobile phase with HILIC is less separation of target analytes; isomer separation for DCCA and DBCA is completely lost. Correspondingly, DCCA and DBCA isomers were quantitated collectively (quantitation results not based on distinct isomers) for development/validation studies.

Ion Suppression/Ion Enhancement

Discussion of extraction efficiency, or specificity, is appropriate here to describe how the overall method performs including sample pre-treatment and ionization. Many analysts often group analyte recovery and extraction efficiency together, however, for the purposes of this research the two will be defined as two distinct parameters. Analyte recovery defines the percentage of target analyte recovered after all sample preparation manipulation. Extraction efficiency defines the percentage of analyte loss in the overall

method including suppression/enhancement effects (sample preparation + ionization efficiency). Often, analytical publications report recoveries greater than 100%; this would be a clear example of the extraction efficiency rather than the recovery of the target analyte (Niessen, Manini et al. 2006).

Ion suppression and/or enhancement affect analyte response signal from the instrument due to components in the matrix that interfere with the ionization process of the target analytes. Ion enhancement and suppression can lead to lower quality methods by distortion of actual target analyte recoveries. In addition, co-eluting components that are not ionized accumulate in the ionization source housing or various source components which can eventually lead to depressed sensitivity of analyte response and/or limited robustness of the overall method. An example of both ion suppression and enhancement are shown in Figure 3.34 below with MDA and DMTP respectively. MDA is slightly suppressed by unknown matrix components while the signal for DMTP is clearly enhanced; the solvent standard signal is twice the signal of the matrix sample. Additionally, it appears that the peak shape of MDA is to some extent warped due to unknown interfering component(s) showing the effect of matrix on chromatography as well as ionization.

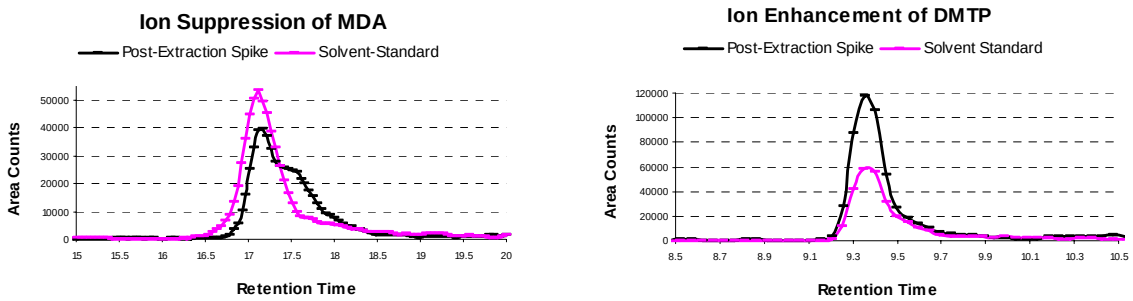


Figure 3.34 Ion Suppression of MDA and Enhancement of DMTP.

Using label internal standards can alleviate matrix effect related problems. It is important to stress, however, that overall matrix effects are not eliminated with label standards but their effects only masked for quantitation. It is logical that by using label internal standards that are the exact same compound as the target analyte (except for the label), the two analytes will behave comparably through sample preparation and ionization. Surrogate standards will behave differently than the target analyte thereby giving different responses. If surrogate standards are used, (and they are used in many methods), it is imperative to use analytes that are most similar in physical structure as well as chemical behavior as that of the target analytes to minimize quantitative error. Decrease in precision is emphasized in Figure 3.35 below that compares the variation in native to internal standard ratio of terbufos sulfoxide (surrogate standard-IMPY label) to the native IMPY with its label IMPY standard in 6 discrete samples.

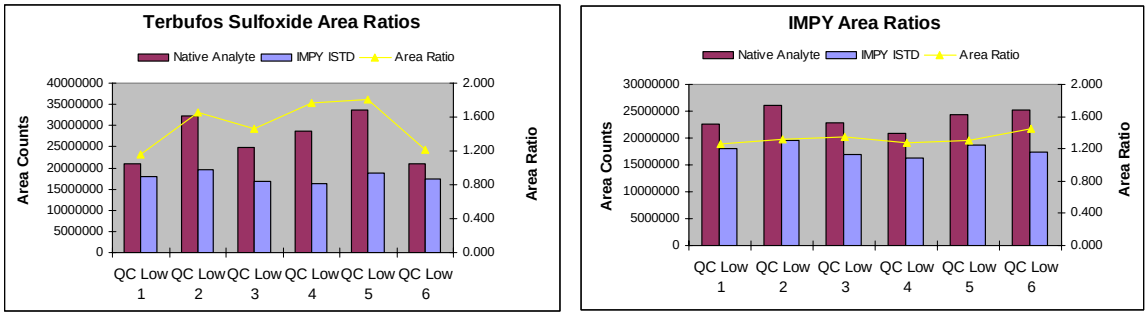


Figure 3.35 Difference in Precision with Terbufos Sulfoxide and IMPY with IMPY-Label Internal Standard.

One way to investigate and possibly reduce matrix effects is to simply change the ion and/or ion-daughter pair being monitored. An example of this is shown in Figure 3.36 below.

CFCA is greatly suppressed by unknown matrix components as shown on the right side of the figure as there is virtually no signal associated with CFCA in matrix monitoring the ion-daughter pair *m/z* 241-121. This is compared to CFCA ion-daughter pair *m/z* 241-205 that has a sufficient signal with no raised baseline. Unfortunately, CFCA is still suppressed by unknown matrix components but the degree of suppression is reduced by simply switching ion pairs being monitored.

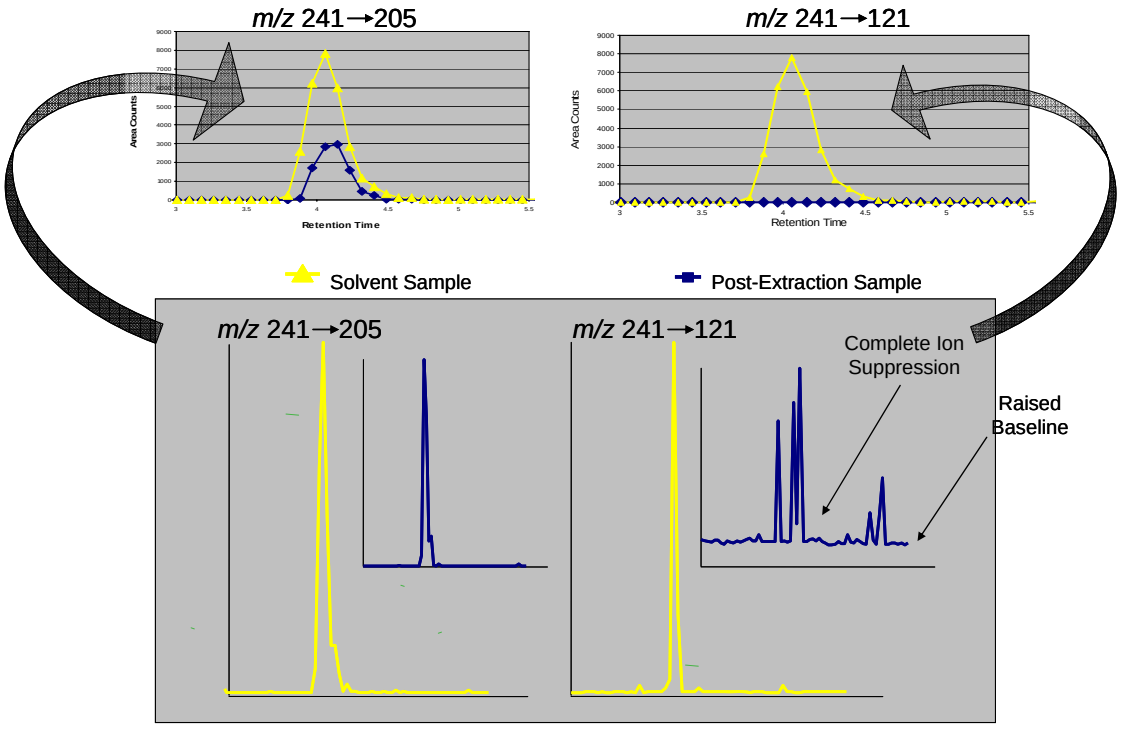


Figure 3.36 CFCA Ion Suppression with 2 Different Parent-Daughter Ion Pairs.

As stated before, matrix effects like ion suppression and enhancement are difficult to completely remove but there are methods to utilize to reduce their effects. As shown with CFCA, simply switching to a different ion-pair can help reduce the effects of matrix components. However, there are still consequences to consider because ion suppression is still present with the ion-pair *m/z* 241-205 in addition to lack of label internal standard

for this target analyte. In addition to label internal standards, preparing standard curves in matrix will help reduce the impact of matrix effects on quantitation because the response ratios will be affected similarly as that of the unknown sample. Even after accomplishing the following steps, matrix effects will still be present and should be accounted for when validating any analytical method. Method robustness is a good indicator for long-term matrix effects impact, *i.e.*, how many samples can be analyzed before instrument maintenance must be performed?

Conclusion

For this method, high recoveries were obtained for all analytes except DMP. Additionally, the LOD calculated for DMP was liberal and should be higher than the 85.668ng/g given. The LOQ for DMP is probably a better estimate for LOD at 285.56ng/g. Although detection limits were much higher than expected for DMP; DMP performed well with the QC validation analysis at concentrations 350ng/g and 550ng/g for QCL and QCH respectively. The low RSDs (<15%) calculated for DMP at these two concentrations indicate that this method can be used to quantitate DMP for this concentration range 350ng/g-550ng/g. Regardless, the likelihood of DMP existing in soil at such high concentrations has not been evaluated but thought to be high as many OP pesticides and DMDTP and DMTP all eventually degrade to DMP as the final end product (Roberts 1999). Therefore, perhaps this method would be useful for analyzing DMP in soil. Lower precision seen with several analytes with high RSDs at the lower end of the standard curve signifies that this method would be semi-quantitative for DBCA, DMDTP, TCPY, CDCA, MDA, sulfones, sulfoxides, IMPY. Low precision seen

at both the low and high-end would indicate that this method is only qualitative for CFCA. Excellent linearity is shown for the target analytes using this method. Additionally, the method is somewhat robust, allowing for an average of ~200 samples to be analyzed before instrument maintenance (ionization source parts cleaning) is required. However, this is far less than other methods reported with polar metabolites and LC-MS/MS analysis (Olsson, Baker et al. 2004; Baker, Olsson et al. 2005). This may be due to inefficient sample pre-treatment and the need for harsher clean-up conditions or perhaps just overall differences with environmental and biological matrices.

Evaluating all method parameters is imperative to determining usefulness of an analytical method. Analyte recoveries can help demonstrate selectivity of a method while detection limits and QC characterization demonstrate sensitivity and the recovery of target analytes free from matrix interferences (usually increased by sample pre-treatment). High recoveries do not always define an acceptable method. There is a trade off between high recoveries and "clean" samples with good precision and accuracy. A harsher sample pre-treatment may reduce the recovery of target analytes. However, harsher clean-ups may reduce interfering components that affect ionization efficiency of target analytes. Logically, the response for an analyte present for ionization and analysis increases as the amount increases. However, this may not be as straightforward as logic decrees. With analytical methodology, sometimes less of the analyte present (with less interfering matrix components) may give better method parameters than a method that has more analyte present (with more matrix components) but more matrix effects thus decreasing overall method validity. As discussed above, ESI sensitivity decreases at sample

concentrations >10-5M. Inclusion of target analytes but exclusion of matrix interferences is the fundamental purpose to achieving successful ionization and therefore quantitation. The balance is delicate and difficult but must be investigated thoroughly for the group of target analytes and the matrix considered.

Higher selectivity for target analytes and lower selectivity for interfering matrix components may help alleviate some of the matrix effect problems encountered with this method. Reasonably, a more selective extraction method may help improve poor precision results and detection limits and increase recoveries for DMP. Therefore, a separate extraction method was analyzed next to determine if higher selectivity can help improve overall method validation parameters needed for a successful, quantitative method.

Sample Preparation – Molecular Imprinted Polymer Solid Phase Extraction

SPE has become extremely popular in the last twenty years for clean-up of complex environmental matrices for semi-selective enrichment of target analytes. SPE methods offer a wide-range of sorbent beds that require relatively low-solvent usage (compared to liquid-liquid extractions) at low cost with easy automation capabilities (Stevenson 1999; Martin-Esteban 2001). Silica-bonded SPE sorbents with C18 selectivity and polymeric-based sorbents are the most commonly used SPE types due to their generic retention mechanisms (Ferrer and Barcelo 1999; Lanza and Sellergren 2001; Pico, Fernandez et al. 2007). General problems found with bonded silica sorbents include sensitivity to extreme pH conditions, limited breakthrough volumes, inability to retain more polar analytes, wettability requirements and secondary interactions with uncapped silanols have been greatly improved with polymeric SPE sorbents (Hennion 1999; Lanza and Sellergren 2001). However, the non-selective feature of these conventional SPE sorbents, silica-bonded and polymeric SPE sorbents, which aid in retaining large groups of chemically divergent analytes, also retains undesirable interferences co-extracted from environmental matrices such as salts and humic acids (Ferrer and Barcelo 1999; Masque, Marce et al. 2001; Le Moullec, Truong et al. 2007; Pico, Fernandez et al. 2007). Trace analysis of target analytes in complex matrices can be complicated by interferences at higher concentrations (Hennion 1999). Interfering co-extracts can lead to undesirable matrix effects which can diminish the quality of an analytical method including inaccurate response, poor precision and robustness. Contrary to analyzing samples with co-extracts present following conventional clean-up, additional clean-up procedure(s) can help further reduce contaminants but can also consequently lead to loss of target analyte

and actually introduce contamination (Hennion 1999). Demands for high-throughput screening with lower detection limits and high precision in combination with extremely complex environmental matrices have increased the necessity for highly selective and sensitive SPE methods (Ferrer and Barcelo 1999; Hennion 1999; Lanza and Sellergren 2001). One-step sample pre-treatment procedures capable of high-throuput analysis are very difficult with conventional SPE extraction with complex matrices (Hennion 1999).

Immunosorbent SPE

In response to this demand, newer class of SPE sorbents such as immunosorbents and enzyme-based sorbents, have been developed that appeal to SPE applications requiring higher selectivity. Immunosorbents are based on affinity interactions between antibodies and target analytes which greatly enhance extraction selectivity of a specific analyte or class of analytes that are structurally similar (Pichon, Chen et al. 1995; Ferrer and Barcelo 1999; Chapuis, Pichon et al. 2004). Additionally, immunosorbents meet the one-step sample clean-up requisite for liquid matrices and secondary purification step for solid matrices (Hennion 1999). Because of the high selectivity with immunochemical extraction, these methods are generally limited to the analysis of one target analyte although recently, analyte-class specific techniques have emerged for pesticide analysis with broad specificity antibodies generated from common haptens (Pichon, Chen et al. 1996; Alcocer, Dillon et al. 2000; Price, Baranowska et al. 2006). However, obtaining antibodies specific for target analytes is difficult, time-consuming and an expensive process that is impractical for high-throughput laboratories (Martin-Esteban 2001). Additionally, they bind irreversibly, limiting cartridge-reuse and have low reproducibility

between batches affecting method precision and analyte recovery (Ferrer and Barcelo 1999; Jenkins, Yin et al. 2001; Lanza and Sellergren 2001; Hennion and Pichon 2003). Enzyme-based methods are less prevalent but have been developed primarily for biosensor development targeting organophosphate and carbamate pesticides with fiber optic bound acetylcholinesterase and butrylcholinesterase (Pandey and Weetall 1995; Andres and Narayanaswamy 1997; Makower, Barmin et al. 1997; Jeanty, Ghommidh et al. 2001; Arduini, Ricci et al. 2005). However, these methods are in the preliminary stages of development and accordingly have not been applied toward real samples. Initial problems seen with enzymatic analyses are problems with enzyme purification, enzyme instability, sensitivity to organic solvents and competition with metal binding, a problem that would be typically encountered with various environmental matrices (Jenkins, Yin et al. 2001; Arduini, Ricci et al. 2005).

Molecular Imprinted Polymer SPE

Because of the problems encountered with immunosorbent and enzyme-based SPE, researchers have synthesized "antibody mimics" that incorporate the high selectivity aspect of biological receptors but without the stability problems and cost constraints (Jenkins, Yin et al. 2001; Lanza and Sellergren 2001; Chapuis, Pichon et al. 2004; Chapuis, Pichon et al. 2004; He, Long et al. 2007). Molecular imprinted polymers (MIPs) are highly selective and stable polymers that possess recognition sites imprinted in the bed of the polymer that are adapted to both three dimensional shape and functionality of the target analyte (Ensing, Berggren et al. 2001; Majors 2007).

Actual reporting of MIP theory initialized in the 1930s in Kiev when a group discovered that silica surfaces have a high affinity for additives that were initially prepared with the silica and subsequently removed and re-exposed (Alexander, Andersson et al. 2006). However, the concept and potential of MIPs for SPE was first realized in 1994 with the development of a selective polymer capable of molecular recognition of pentamidine in urine, a drug used for AIDS-related pneumonia (Sellergren 1994; Ferrer, Lanza et al. 2000). Since 1994, the interest in MIPs has risen exponentially as shown below in Figure 3.37.

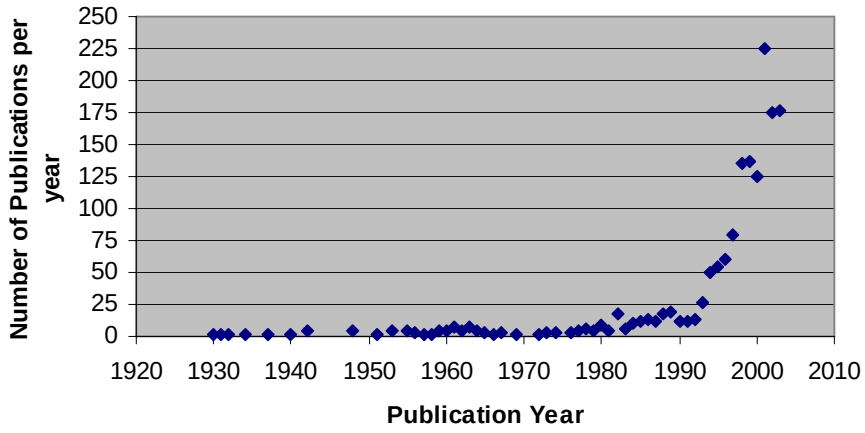


Figure 3.37 Number of MIP-Related Publications Since 1930 *Adapted from* (Alexander, Andersson et al. 2006).

Most research groups manufacture their own MIPs specific to their research because the synthesis is fairly simple and relatively inexpensive. Recently, five start-up companies: MIP Technologies (Lund, Sweden), Affinity Chromatography, Ltd. (Isle of Man, UK), Aspira Biosystems, Inc. (San Francisco, CA), Ellipsa AG (Berlin, Germany) and Instruction AG (Ludwigshafen, Germany) have emerged that propose to develop custom

made MIP-based SPE (MISPE) phases (Majors 2007). As such, the availability of MISPE phases has contributed to the increase seen in publications.

There are several methods to create MIPs but the basic steps follow this pattern (Sellergren 1994):

1. copolymerization of functional monomers that surround the template containing the target analyte with a cross-linker monomer
2. displacement of the template containing the target analyte from the polymer which leaves behind binding sites ("imprinted") complementary to the target analytes
3. investigation of the copolymer for molecular recognition of the template target analytes

There are several methods to assemble the polymer-template construct including: covalent binding, non-covalent binding, semi-covalent, metal-binding and metal-mediated (Alexander, Andersson et al. 2006). For the purposes of this report, only the non-covalent approach will be discussed here as it is the most widely used method to create imprinted polymers for SPE purposes and it has more utility for SPE in the future due to vast numbers of analytes capable of noncovalent interactions with polymerizable monomers (Ensing, Berggren et al. 2001; Masque, Marce et al. 2001; Chapuis, Pichon et al. 2004; Alexander, Andersson et al. 2006; Qiao, Sun et al. 2006; He, Long et al. 2007; Majors 2007). Figure 3.38 below illustrates the synthesis of a MIP for atrazine recognition.

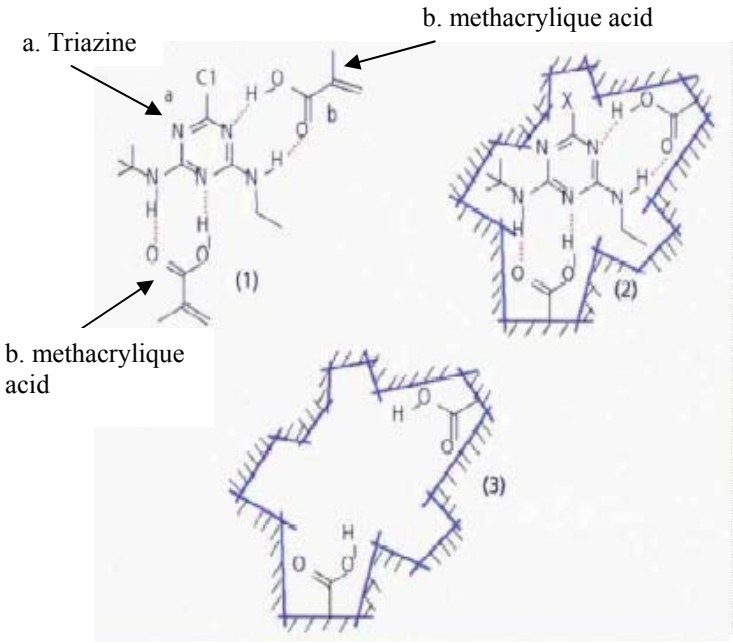


Figure 3.38 MIP Synthesis *Used with permission* (Chapuis LCGC).

Synthesis of a non-covalent MIP

1. Formation between polymers and template with porogenic solvent
2. Polymerization of functional monomers with cross-linkers around template
3. Removal of template and formation of complementary binding sites

Selective noncovalent interactions between the template and the monomers are based upon hydrogen, ionic and hydrophobic bonding (Ensing, Berggren et al. 2001). The most common monomer used for MIP synthesis is methacrylic acid as shown in Figure 3.38, and together with the template is commonly crosslinked with ethyleneglycol dimethacrylate or divinylbenzene (Lanza and Sellergren 2001; Alexander, Andersson et al. 2006; Majors 2007). The "tailor-made" template generated by this process gives MISPE a substantial advantage over other conventional sorbents because of their high selectivity.

Not only does MISPE offer much higher selectivity than conventional SPE (silica and polymer based), but is also flexible to method requirements-it can actually be used with

both aqueous and organic solvents depending upon target analyte specificities (Ensing, Berggren et al. 2001; Qiao, Sun et al. 2006). Other advantages include high stability with pH extremes and increased temperatures (up to 120º C) and lower cost and faster production times relative to immunoaffinity columns (Ensing, Berggren et al. 2001). All of the advantages evident with MISPE clearly indicate a new and exciting frontier for separation science. However, as the utility for MISPE is still in the early stages, there are still disadvantages to overcome.

The most widely discussed disadvantage is the possibility of target analyte leaching during use (Ensing, Berggren et al. 2001). Incomplete removal of the template from the polymeric sorbent leads to analyte "bleeding" during MISPE, which can lead to quantitation errors, especially at low levels (Lanza and Sellergren 2001; Masque, Marce et al. 2001; Chapuis, Pichon et al. 2004; Majors 2007). The degree of bleeding has not been measured, although it is estimated that more than 1% template remains even after excessive washing (Lanza and Sellergren 2001). To rectify the leaching dilemma, a label or target analyte analog ("dummy template") has been used as the imprint to retain target analytes similar in structure and chemical behavior but without compromising quantitative results (Chapuis, Pichon et al. 2004; Majors 2007). The analog imprint approach, first published in 1997 (Andersson, Paprica et al. 1997) has worked successfully for several analytical methods resulting in methods that are of higher quality than conventional SPE methods (better precision, lower detection limits and higher accuracy). For example, Kawaguchi *et.al.*, synthesized a MISPE containing bisphenol A-d_{16} for the detection of bisphenol A in river water (Kawaguchi, Hayatsu et al. 2005).

Using bisphenol A-$^{13}C_{12}$ as surrogate standard, the group was able to achieve 99.8% recovery (RSD 3.7%) with an extremely low detection limit of 1pg/mL (ppt). Although these results show excellent analytical quality, the use of 2 label standards per target analyte can be extremely costly and difficult to locate making this method impractical or more suitable to only 1 or a few analytes. Alternatively, Mullett *et.al* used an analog compound (2-aminopyridine) as the imprint template for the extraction of 4-aminopyridine from human serum and had successful results with a detection limit of 52ng/mL (ppb) and 82% recovery (5.1%RSD) (Mullett, Dirie et al. 2000).

Several publications have demonstrated differences between conventional SPE extraction and MISPE (Andersson, Paprica et al. 1997; Blomgren, Berggren et al. 2002; Fiori, Civitareale et al. 2005; Schirmer and Meisel 2006). For example, Figure 3.39 below clearly shows the reduction in baseline and matrix interferences indicated by numerous peaks for the extraction of clenbuterol from calf urine samples with a brombuterol-imprinted MIP compared with three various conventional non-imprinted mixed-phase polymers (Blomgren, Berggren et al. 2002; Widstrand, Yilmaz et al. 2006).

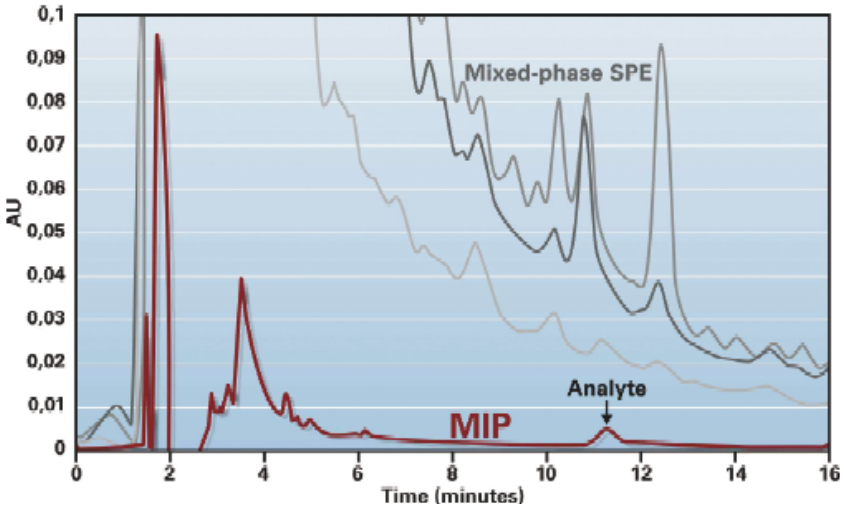


Figure 3.39 MIP Extraction vs. Conventional Extraction of Clenbuterol from Calf Urine *Used with permission* (Blomgren, Berggren et al. 2002).

Method performance parameters investigated include: analyte recovery (75%), within-day precision (4.3% for 0.6ng/mL and 2.1% for 6.0ng/mL), between-day precision (6.4% for 0.6ng/mL and 4.4% for 6.0ng/mL) and accuracy (96.7% for 0.6ng/mL and 96.7% for 6.0ng/mL) and selectivity (see Figure 3.40 below) measurements. The selectivity test was performed after loading of analyte onto the brombuterol-MISPE and a non-imprinted conventional polymer SPE and subsequent elution of 1mL acetonitrile with increasing amounts of acetic acid. Results indicated that more acetic acid was required to break interactions between MISPE-target analyte and non-imprinted-target analyte.

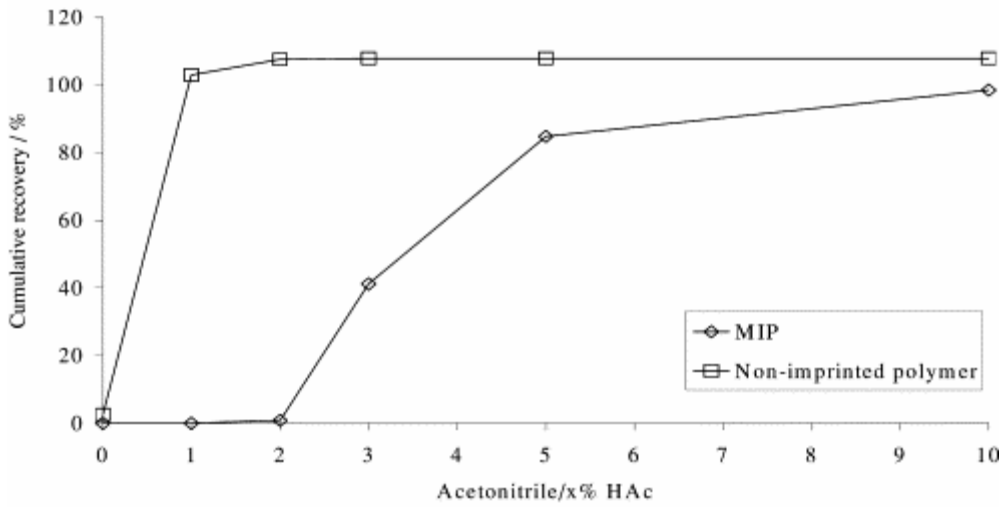


Figure 3.40 Elution of Clenbuterol with Varying Amounts of Acetic Acid in Acetonitrile *Used with permission* (Blomgren, Berggren et al. 2002).

This test indicates a fundamental difference between MISPE and conventional (non-imprinted) SPE polymers. The conventional polymer typically exhibits one type of interaction whereas the MIP exhibits multiple interactions (ionic, hydrogen, hydrophobic) and complementary steric binding specific to the target analyte that increase overall retention to the MIP sorbent (Blomgren, Berggren et al. 2002). Thus, the MIP can be washed under harsher conditions to remove co-extracts compared to the non-imprinted polymer. This is highly advantageous for trace analysis requiring high quality (good precision and accuracy) with low detection limits free from complex matrix interferences.

Environmental Studies with MISPE

Although most of the early research utilizing MISPE has been completed with biological analysis, interest has recently increased for environmental applications. As of May 2006, 35.52% of MISPE applications have been developed for environmental purposes as shown in Figure 3.41.

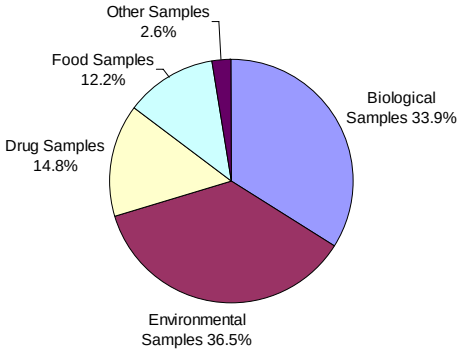


Figure 3.41 Percentages of MIPSE Studies by Application *Adapted from* (He, Long et al. 2007).

The majority (29/43) of the MISPE environmental applications have targeted analytes either off-line or on-line in river water or other environmental waters in the past 5 years (He, Long et al. 2007). Additionally, there have been 9 MISPE-soil/sediment methods published in the past 5 years with 5/9 targeted toward pesticide class analysis instead of single analyte analysis (He, Long et al. 2007; Le Moullec, Truong et al. 2007).

Despite the high selectivity offered with MISPE, cross-reactivity can occur which can actually contribute to applications for screening multiple analytes that are chemically and/or physically similar (Ferrer, Lanza et al. 2000; Masque, Marce et al. 2001). Specifically for pesticide analysis, the advantage of cross reactivity will allow for multiple pesticides within a class to be analyzed at one time since they are typically similar in physical structure and chemistry. Additionally, in consideration of practical pesticide application, typically pesticides are present in environmental matrices as mixtures of several compounds (Ferrer, Lanza et al. 2000).

The feasibility of MISPE for pesticide class extraction has been demonstrated by the selective extraction of the chlorotriazine and thiotrazine herbicides with a terbutylazine-imprinted MIP from sediment and water samples (Ferrer, Lanza et al. 2000). The group also investigated if the triazine-imprinted MIP could extract other pesticide classes similar in structure (phenylureas). However, results indicated that the MIP provided only non-selective retention of phenylurea herbicides with no recovery after a wash step with dichloromethane. The triazines pesticide class are either halogenated- or thio-diamino-triazines whereas the phenylurea typically consist of a benzyl diamide backbone that have mono- or di-substituted attachments as shown in Figure 3.42 below.

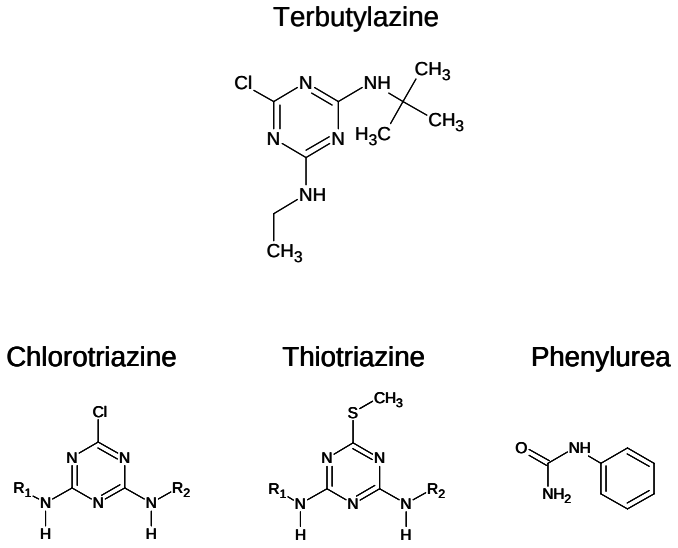


Figure 3.42 Structures of Typical Active Ingredients for Triazines and Phenylureas Pesticides.

Thus, the triazine-MIP was capable of distinguishing between the two somewhat similar pesticide classes. Not only was the MIP successful at retaining multiple herbicides within a class with MIP-specific interactions but also many interferences (including other pesticides that are structurally similar) were able to be removed from samples by disrupting non-selective interactions thus resulting in a cleaner sample (as proven with

the dichloromethane wash step that removed the phenylurea herbicides). A cleaner sample will yield better quality data and therefore desirable quantitation parameters.

Another group showed different results for an organophosphorus (OP) application. Zhu *et.al*. developed a monocrotophos (MCP)-imprinted MIP for the extraction of 4 OP insecticides (MCP, mevinphos, phosphamidon, omethoate) and determined that the MCP-MIP was not selective for other OPs (phorate, dimethoate, diazinon, fenitrothion, parathion) (Zhu, Yang et al. 2005). Therefore, they proved that the MIP they had manufactured would not be useful for OPs as a class but only selective toward a few. Figure 3.43 below shows the structural similarities and differences between the targeted OPs.

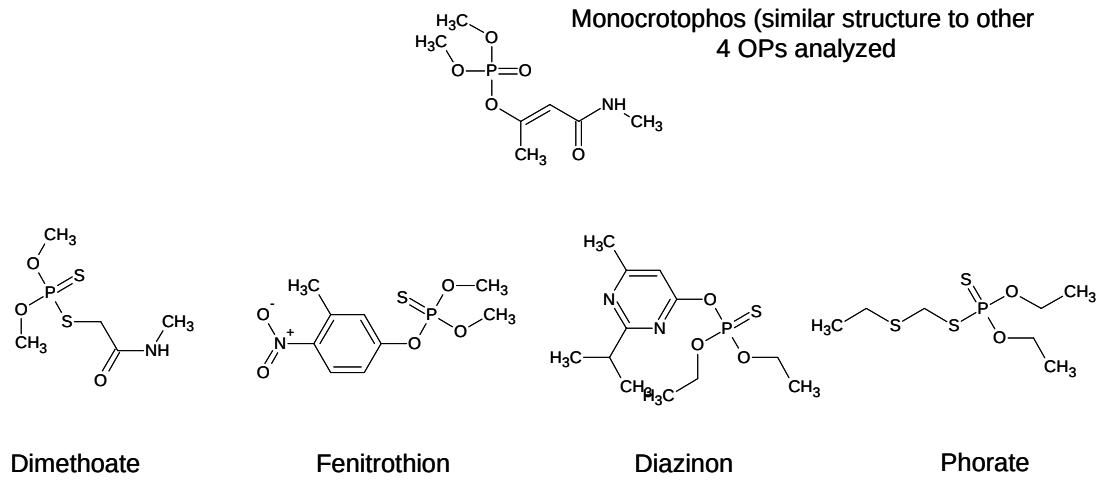


Figure 3.43 Structural Differences between MISPE Imprinted with Monocrotophos and 4 OPs.

It is not surprising that diazinon and fenitrothion did not behave similarly to the target OPs considering the presence of the bulky benzene group with fenitrothion and pyrazine group with diazinon. However, phorate and dimethoate are both structurally similar to the target OPs, although they are thio-containing organophosphate compounds. Perhaps

the sulfur-containing compounds (-P=S) would exhibit less retention with the MIP as compared with (–P=O) compounds due to weaker hydrogen bonding. Specifically, hydrogen bonding was identified as the most significant interaction for retention with this particular MIP (Zhu, Yang et al. 2005). Figure 3.44 below shows the difference between extractions with a conventional SPE sorbent (b, c) and the constructed MIP (d, e) as compared to a standard solution (a).

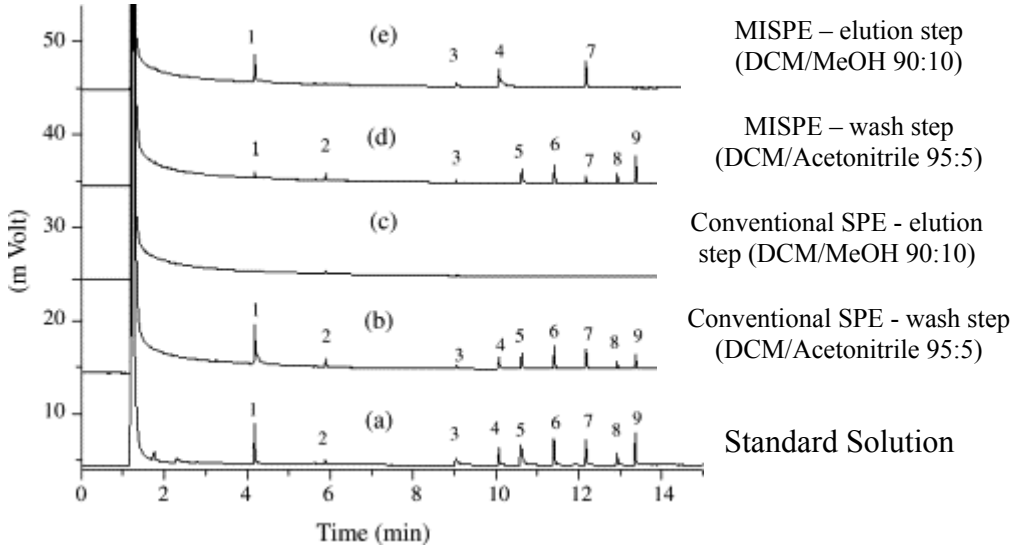


Figure 3.44 Extraction Differences between Conventional SPE and MISPE for Various Pesticides (1-Mevinphos, 2-phorate, 3-omathoate, 4-MCP, 5-dimethoate, 6-diazinon, 7-phosphamidon, 8-fenitrothion, 9-parathion) *Used with permission* (Zhu, Yang et al. 2005).

Figure 3.44 above shows a very good illustration of the difference between conventional and MISPE. With the high organic wash, all of the target analytes (1, 3, 4, 7) are eluted from the conventional sorbent in comparison to the MISPE where a portion of the target analytes are eluted with the wash solvent but the majority are retained for the final elution step. This shows the higher selectivity component associated with MISPE. Although recoveries may be less (MCP-83% with MIP compared with 98% with conventional

SPE), samples were reported as cleaner overall due to the higher selectivity (Zhu, Yang et al. 2005).

Pesticide Degradation Products in Soil with MISPE

Although recoveries for the degradation products in soil using the ion-exchange extraction were high (except DMP), the precision was poor for many of the target analytes, particularly at the lower end of the calibration curve (CFCA, DBCA, DMDTP, TCPY, CDCA, MDA, sulfones, sulfoxides, IMPY). These problems are assumed to be due to matrix effects which can greatly lower quality of analytical methods. To reduce possible matrix effects, MISPE was investigated to improve overall selectivity of target analyte extraction and thus method quality control measures.

In March 2006, MIP Technologies launched a new product line, ExploraSep™, a library of sorbents that provides selective screening for separation applications (LaboratoryTalk 2006). Exploiting the cross reactivity inherent with imprinted polymers, the product line, similar to drug screening libraries in the pharmaceutical industry, is utilized to screen targets analyte(s) against MISPE sorbents with varying imprinted molecules and functionalities (Billing 2006; LaboratoryTalk 2006). The disadvantage to using this product is the strict proprietary information of the sorbent chemistry under investigation as the method was evaluated in partnership with MIP Technologies. Therefore, the method development normally implemented with any analytical method is controlled by the company as the users have no knowledge of the underlying MISPE mechanism. All

method development steps performed were dependent upon instructions received from MIP Technologies.

Method Development

MIP Technologies provided 156 sorbents specified with various monomers with acidic, basic, neutral or "special" functionalities in combination with cross-linkers having hydrophilic or hydrophobic properties in addition to the specific imprint itself. These sorbents were screened against the degradation products specified in this research to determine if any "hits" were made; "hits" indicating a significant percentage of target analyte(s) bound to the sorbent(s) under investigation.

Target analytes in buffered water at pH 7 (the solvent of initial soil extraction) were passed through each MIP sorbent in triplicate to determine break-thru with various sorbents. The percentage of analyte break-thru, normalized to 100% recovery samples (samples not passed through the sorbents), determined if any analyte was bound to any particular sorbent and thus if any "hits" occurred. Unlike previous method development, the specific goal of this first step was to find low recoverable samples as this would indicate "hits". Normalized recoveries were subtracted from 100% to determine percentage bound to the MIP(s) sorbent. Two examples (DCCA and DEDTP) of initial screening results are shown below in Figure 3.45.

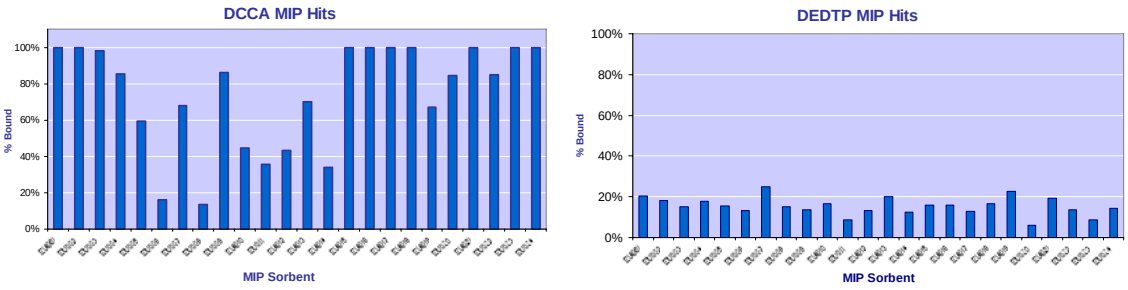
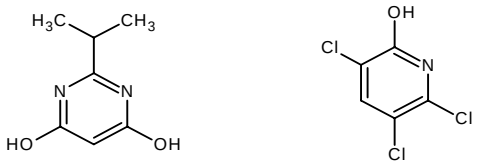


Figure 3.45 DCCA and DEDTP Hits with Various MISPE Sorbents.

DCCA resulted in many hits in comparison with DEDTP that had no hits with this particular group of MIP sorbents tested. Overall, there were many hits with the pyrethroid and specific organophosphate degradation products; however; there were no significant hits with any of the DAPs for any of the MIPs tested. The poor binding exhibited with the DAPs is difficult to explain because the chemistry of the sorbents is unknown. Perhaps the physical size of the small, alkyl chain DAPs compared with other analytes that are larger alkyl chains or aromatic structures hinders binding to the imprinted cavities of the MISPE sorbents.

Additionally, IMPY resulted in low percentage binding (15-50%) for all MIPs tested. Reasoning for why IMPY exhibited lower binding in comparison with the other analytes is even more difficult to explain. IMPY is a dihydroxy pyrimidine derivative similar in structure to TCPY which exhibited 100% binding for many of the sorbents. The structures of the two analytes are shown in Figure 3.46 below.



IMPY TCPY

Figure 3.46 Structures of IMPY and TCPY.

Evaluation of all following method development steps were continued with hit MISPE sorbents only. The next step involved altering loading pH to attempt to improve retention for the analytes. Although the DAPs are ionic over the pH range3-7, lowering the pH (to 3, 3.5 and 4) versus pH5-7 greatly improved recoveries for the DAPs with the ion exchange extraction seen in Table 3.10. If there is an ion exchange component with any of the selected MISPE, perhaps lowering the pH could help improve retention of the DAPs. Loading solvent (buffer pH7) containing target analytes was acidified to either pH 1, 2, or 3 with formic acid before sample loading onto MISPE sorbents. Analyte break-thru was collected and normalized to 100% recovery samples not passed through the cartridges. Results are shown below in Figure 3.47 for DMP and DEP.

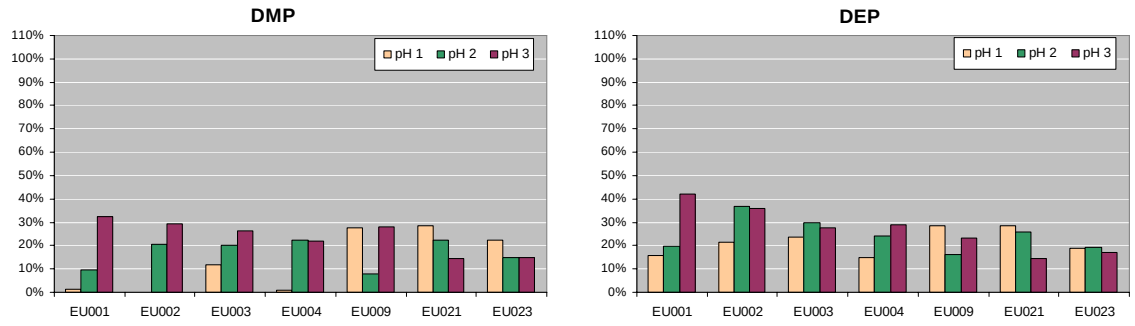


Figure 3.47 Retention Behavior of DMP and DEP with Selected MISPE Sorbents at pH1-3.

Lowering the pH did not improve retention of the DAPs for any of the selected MISPE. This may indicate that the interaction of the MISPE with the target analytes is not an ion exchange mechanism. However, alteration of the pH *did* improve retention of several of the other target analytes as shown with 4-NP in Figure 3.48.

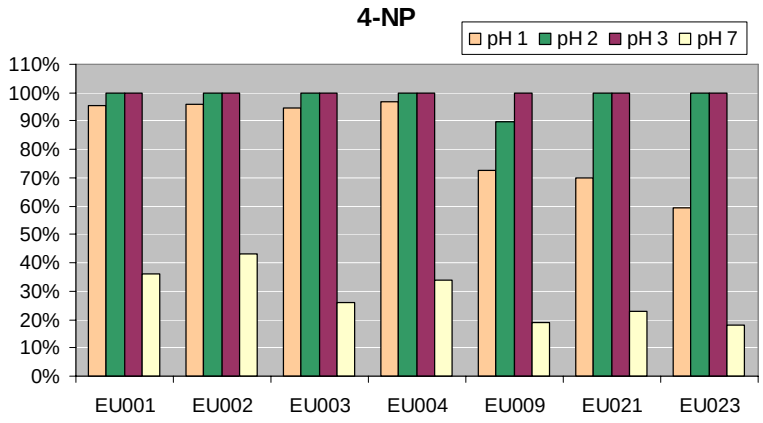


Figure 3.48 Retention Behavior of 4-NP with Selected MISPE Sorbents at pH1-3.

Surprisingly, 4-NP had much better retention at lower pH, although less retention resulted at pH 1 versus pH 2 and pH 3. Collectively, all of the target analytes, excluding DAPs, had the highest retention at pH 3, which may indicate that ion exchange is involved, albeit with a different mechanism than the previous ion-exchange method investigated.

The next step for MISPE analysis was determination of appropriate elution solvent to remove target analytes from MISPE sorbents. Target analytes in a "harsher" solvent of acetonitrile/water/triethylamine (96:3:1) were applied to the MISPE sorbents to encourage analyte break-thru (as opposed to retention with earlier steps). The purpose of this step was to establish an eluent, or elution solvent harsh enough to break the interactions between target analytes and MISPE sorbent. An example is shown below

with DCCA and CFCA in Figure 3.49. It is important to note here that analyte break-thru was normalized to 100% recovery samples and measured as % recovery (as opposed to % bound with earlier steps).

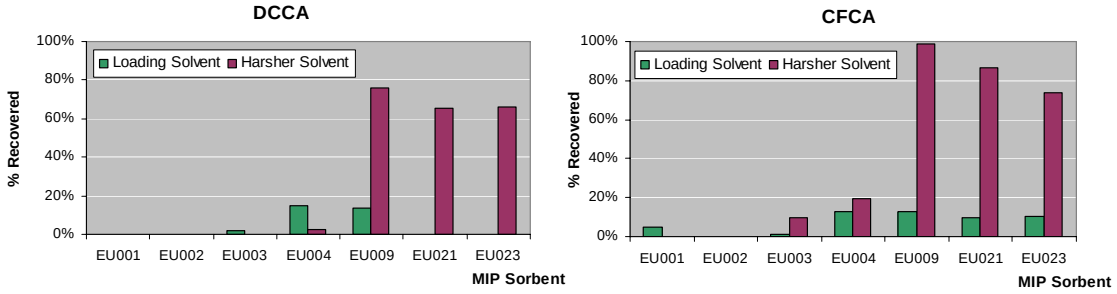


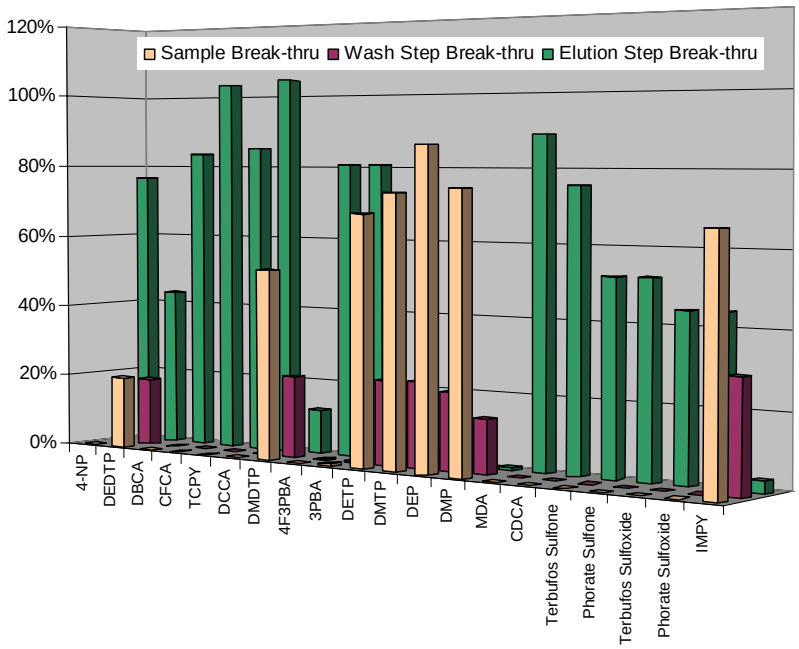
Figure 3.49 Elution Step and Break-thru Analysis of DCCA and CFCA.

As shown in Figure 3.49 above, DCCA and CFCA are still retained to several of the MISPE sorbents (EU001-EU004). In contrast, for MISPE EU009, EU021 and EU023, the majority of DCCA and CFCA are eluted from the sorbents. These results indicate that the harsher solvent is capable of disrupting interactions between these two analytes and MISPE sorbents EU009, EU021 and EU023 and thus would be an appropriate solvent for the elution step. For the target analytes collectively, MISPE sorbent EU009 had the overall highest percentage of analytes bound with the loading solvent (buffer water at pH 7) and the highest elution recoveries with the elution solvent acetonitrile/water/triethylamine (96:3:1). Therefore, all further analysis was carried out with MISPE EU009.

The next step investigated was the wash step to ensure no target analyte break-thru occurred while attempting to wash the sorbent free from co-extracting interferences. Two wash solvents were investigated: 1. deionized water (pH 3) and 2. pH 3 deionized water and 0.1M ethylene diamine tetracetic acid (EDTA) (1:1). EDTA was integrated

into the wash step to remove any metal ions present in soil that may interfere with either retention to the sorbent or instrumental analysis due to matrix effects. To investigate analyte break-thru for the entire process, break-thru solvent was collected after each step (loading, wash, and elution) and normalized to 100% recovery samples. Complete elution behaviors of all target analytes are shown in Figure 3.50 below.

A. Wash: pH 3 DI water



B. Wash: pH 3 DI water/0.1M EDTA (1:1)

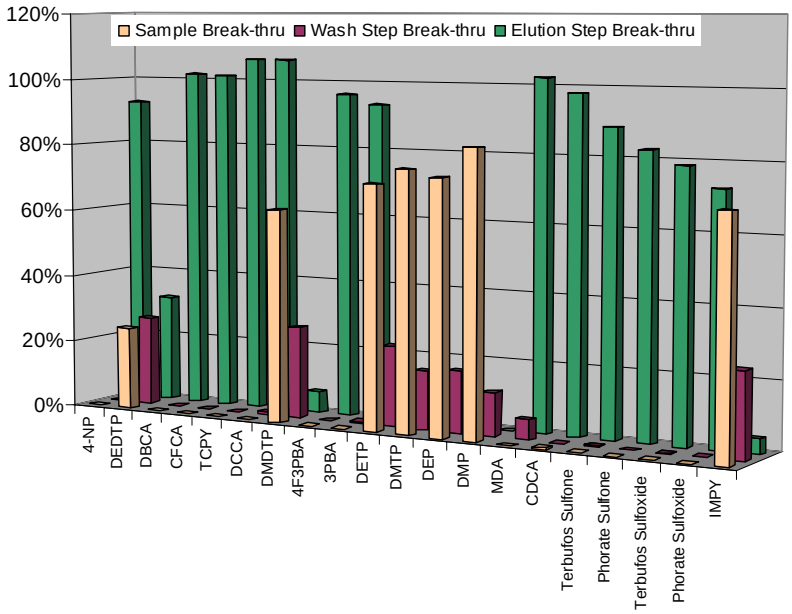


Figure 3.50 Break-thru Profiles for Target Analytes Comparing 2 Different Wash Steps (A-pH 3 dH_20, B-pH 3 dH_20/0.1M EDTA (1:1)).

The above figures detail target analyte break-thru patterns for sample loading, wash step and elution step with the wash step as the only variable between the two figures (A and B). IMPY and the DAPs exhibit the same pattern for both A and B, with most of the analyte breaking thru with the loading solvent, leaving less than 20% bound to the sorbent and even less bound after the wash step. Interestingly, elution recoveries with the pH 3 DI water/0.1M EDTA (1:1) wash step were higher than with the pH 3 DI water wash alone. This is surprising as the inclusion of EDTA with the wash step for this analysis was performed for the purpose of ensuring no sample loss and any increases in recovery were not expected. The increase in recoveries with the addition of EDTA to the wash solvent would be more logical if these were real soil samples because metals could metal(s) intrinsic to soil could compete with SPE binding sites. However, these were

solvent standards without matrix metals and the addition of EDTA to the washing solvent was expected to have minimal effect on analyte recoveries. Perhaps the EDTA improves recoveries of target analytes by removing interfering ions from the phosphate buffer and formic additive that may have possibly retained on the MISPE sorbent thus allowing the elution solvent to interact with target analytes more effectively.

Due to lack of binding of the DAPs with the available MIP sorbents, they were excluded from further study. Because LODs for IMPY were expected to be low with MISPE (due to extremely low LODs with the ion-exchange mechanism) it was included for further study although recoveries were expected to be lower than other target analytes. Thus, the final MISPE method chosen to continue with method validation is shown in Figure 3.51.

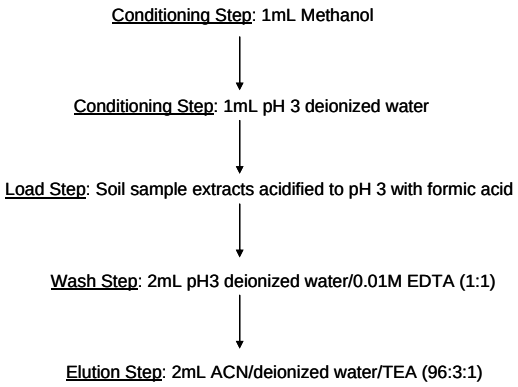


Figure 3.51 Flowchart of MISPE Steps.

Method Validation

The same parameters investigated for the degradation products with the ion-exchange method will also be discussed here. The same standard concentrations used for the

validation of the ion-exchange method were used for validation of the MISPE method unless otherwise indicated (Table 3.12).

Detection Limits

The exact procedure used to determine method detection limits for the ion-exchange method was also used for the MISPE method. Table 3.16 below shows method detection limits for the MISPE method in comparison with the ion-exchange method.

Degradation Products Method Detection Limits

	MISPE		Ion-Exchange	
	LOD	LOQ	LOD	LOQ
4-NP	2.50	8.32	2.33	7.76
DBCA	3.57	11.90	5.00	16.66
CFCA	1.76	5.86	4.97	16.56
DCCA	9.84	32.80	28.90	96.34
TCPY	5.02	16.73	10.32	34.39
3PBA	1.15	3.84	0.33	1.11
4F3PBA	0.66	2.20	0.59	1.98
CDCA	2.38	7.92	18.87	62.89
MDA	2.05	6.83	8.42	28.08
Terbufos Sulfone	1.34	4.45	3.34	11.13
Phorate Sulfone	2.48	8.26	3.90	12.99
Terbufos Sulfoxide	0.89	2.98	6.70	22.35
Phorate Sulfoxide	1.59	5.31	2.65	8.84
IMPY	2.36	7.86	0.13	0.42

Table 3.16 Comparison of Limit of Detection and Quantitation for Target Analytes with MISPE or Ion-Exchange SPE.

Collectively, all target analytes had lower detection limits with the MISPE method as compared to the ion-exchange method (excluding 3PBA and IMPY). It is not surprising that IMPY had a higher detection limit considering the lower recovery with this method. The SN ratio of IMPY at S1 (2ng/g) is 11(Figure 3.52), which confirms that an LOD of 2.36 is slightly conservative.

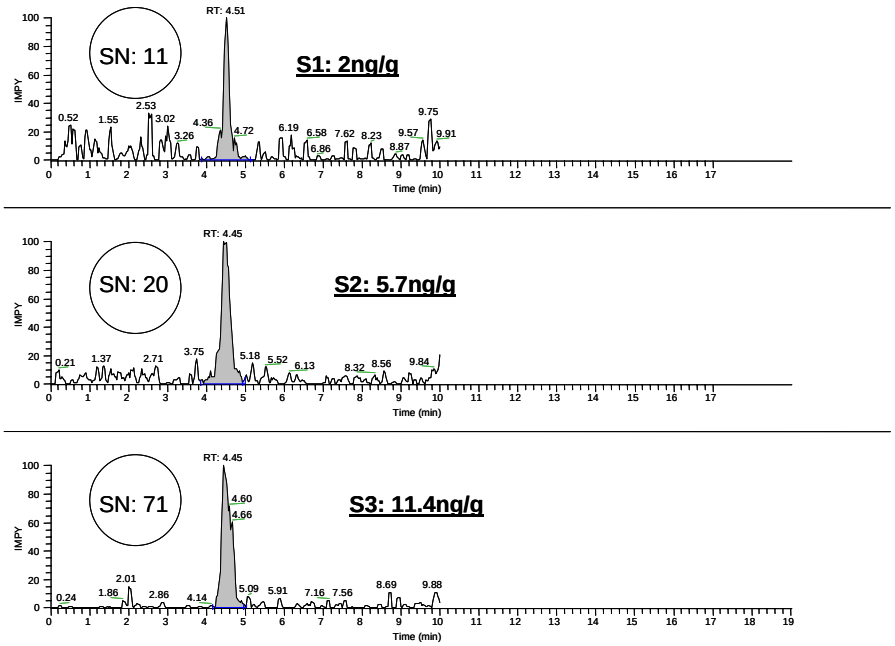


Figure 3.52 SN Ratio of IMPY at Different Concentrations.

Like IMPY, all of the LODs calculated for the target analytes were either slightly conservative or comparable to the SN ratios determined from the chromatograms; no underestimation of LOD values were observed as seen with DMP with the ion-exchange method. The lower LOD values observed for most of the target analytes support previous MISPE research that conclude that MISPE is a more sensitive extraction technique than other SPE techniques.

Analyte Recoveries

Again, the exact procedure for analyte recovery determination for the ion-exchange method was also performed with the MISPE method (S4 and S7). Figure 3.53 below shows overall method and SPE recoveries for the target analytes analyzed. Figure 3.54

below shows overall recovery comparison at S7 for the ion-exchange and MISPE methods.

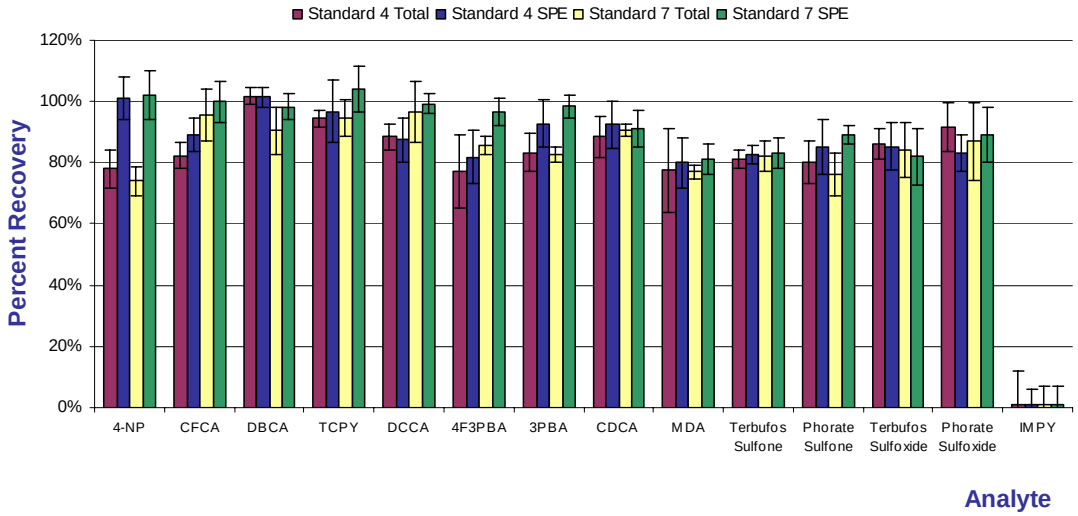


Figure 3.53 Percent Recovery for Target Analytes with MISPE Method at S4 and S7.

Generally, recoveries for the target analytes using MISPE were very high (78%-102%) excluding IMPY (4%). Recoveries were also consistent between low (S4) and high (S7) concentrations.

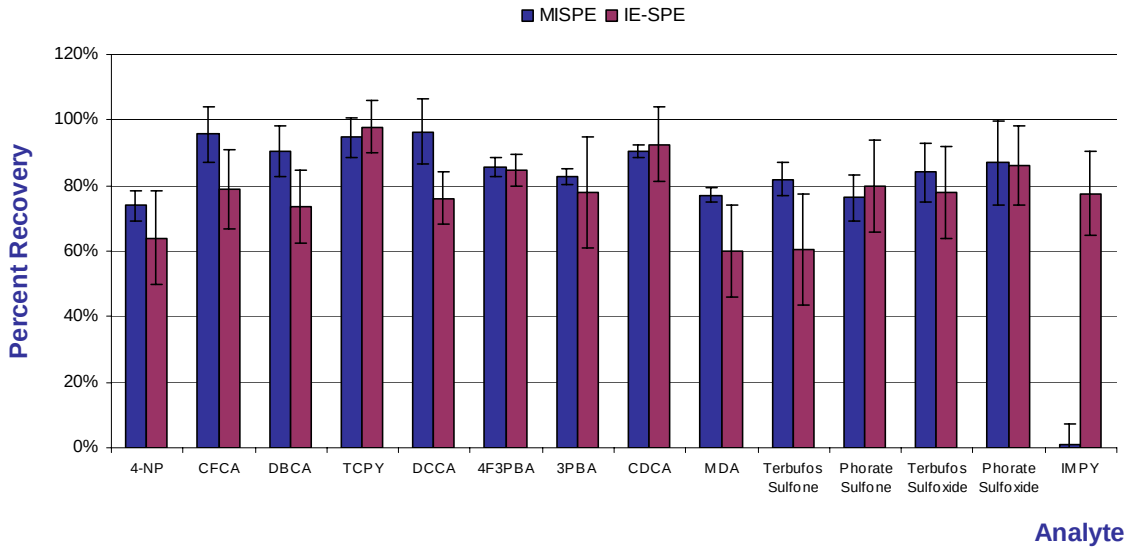


Figure 3.54 Percent Recovery Comparison between MISPE and Ion-Exchange SPE at S7.

Overall, analyte recoveries using the MISPE method were slightly higher than with the ion-exchange method excluding IMPY which yielded much lower recoveries (4%) with the MISPE method (78% for the ion-exchange method).

Quality Control Characterization

QC materials were prepared in the same procedure as the ion-exchange method with the exception of DCCA that was spiked at the same concentration as the other target analytes (QCL-25ng/g and QCH-100ng/g) due to lower detection limits found using MISPE. The following Table 3.17 summarizes accuracy and precision calculations with QC results.

Analyte	Accuracy		Precision (RSD)					
	Average (%dev)		Within Day		Between Day		Overall	
	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*
PNP	22.6 (-9.6)	98.4 (-1.6)	8.1	5.5	12.2	5.4	14.1	12.3
DBCA	26.2 (4.8)	101.2 (1.2)	5.8	4.0	9.8	4.2	13.0	9.0
CFCA	26.3 (5.2)	90.1 (-9.9)	9.6	6.7	13.2	6.6	21.4	14.9
DCCA*	24.4 (-2.4)	102.1 (2.1)	4.7	5.4	14.2	11.5	10.4	12.0
TCPY	22.5 (-10.0)	81.2 (-18.8)	4.2	2.9	7.6	2.1	9.3	6.6
3PBA	25.9 (3.6)	76.0 (-24.0)	4.3	3.0	5.6	5.4	9.7	6.7
4F3PBA	21.9 (-12.4)	65.4 (-34.6)	4.7	3.1	7.5	7.0	10.5	6.8
CDCA	26.4 (5.6)	100.3 (0.3)	8.3	5.9	15.7	8.5	18.5	13.2
MDA	25.6 (2.4)	72.0 (-28.0)	8.5	6.2	6.4	9.7	9.1	9.8
Terbufos Sulfone	24.8 (-0.8)	72.6 (-27.4)	6.5	4.5	5.3	6.4	14.5	10.2
Phorate Sulfone	24.7 (-1.2)	75.2 (-24.8)	6.6	4.6	11.1	14.7	14.7	10.2
Terbufos Sulfoxide	27.2 (8.8)	73.6 (-26.4)	6.6	5.9	12.4	4.4	14.7	13.2
Phorate Sulfoxide	25.4 (1.6)	72.6 (-27.4)	6.6	4.9	6.3	5.0	14.1	11.1
IMPY	25.4 (1.6)	71.0 (-29.0)	7.8	6.6	24.2	10.2	17.3	14.8

* DCCA spiked at 25ng/g (QCL) and 100ng/g (QCH)

Table 3.17 Accuracy and Precision Summary for Degradation Products with MISPE Method.

The average within-day and between-day RSD for QCH measurements was 4.9% and 7.2% respectively. The average RSD was slightly higher at the QCL level for within-day (6.6%) and between-day (10.8%). However, overall average RSD was similar for both QCH (10.8%) and QCL (13.7%). All target analytes had individual, overall RSDS ≤

16% (QCH). Only 3/14 target analytes had individual, overall RSDs ≥ 16% at the QCL level (CFCA, CDCA, IMPY).

Average RSDs found for the ion-exchange method at both low and high concentrations are approximately twice that of the MISPE method for within-day, between-day and overall (QCL) precision measurements as shown in Table 3.18. The overall QCH RSD for MISPE was slightly lower (10.8%) than the QCH RSD for the ion-exchange method (13.7%).

Average RSD Comparison Between Ion-Exchange and MISPE (%)

	Ion-Exchange*		MISPE	
	QCL	QCH	QCL	QCH
Within-Day	14.7	14.0	6.6	4.9
Between-Day	20.3	14.2	10.8	7.2
Overall	25.6	13.7	13.7	10.8

*Recalculated for pyrethroid and non-specific organophosphorus degradation products only

Table 3.18 Average RSD Comparison between MISPE and Ion-Exchange.

There is no question that the MISPE method gives more precise measurements than the ion-exchange method for the pyrethroid and non-specific degradation products. Problematic target analytes with the ion-exchange method (high RSDs) such as the sulfones/sulfoxides all yielded lower RSDs with the MISPE method at the QCL level. As a result, the MISPE method could be used for quantitation purposes toward the lower end of the calibration curve for these analytes as compared to the ion-exchange method where only the QCH level was within acceptable range. Both CDCA and CFCA had lower RSDs at both QCL and QCH level for the MISPE method; however, RSDs calculated for QCL were still too high for quantitation (≥ 16%) and therefore still limited to qualitative

assessment only. The RSDs calculated for IMPY at the QCL level for the MISPE method were similar to that seen with the ion-exchange method (17.3%-MISPE compared with 17.1%-ion-exchange). At the QCH level, IMPY had higher RSDs for the MISPE method (14.8%) as compared with the ion-exchange method (9.4%). This could be due to the low recovery and higher LOD found for IMPY using the MISPE method.

The accuracy measurements for the MISPE yielded unexpected results in that all target analytes at the QCL level are within +/-15% of the nominal spiked value; however, almost all analytes (9/14) at the QCH level exceeded +/-15% of the nominal spiked value. All of the analytes that exceeded the +/-15% limit were all lower than the spiked value (100ng/g). A similar trend was observed for the ion-exchange method as shown in Table 3.19 where target analytes that exceeded the limit were well below the spiked value.

Accuracy Comparison Between Ion-Exchange and MISPE

Analyte	Average (%dev)			
	Ion-Exchange		MISPE	
	QCL *n=36*	QCH *n=36*	QCL *n=36*	QCH *n=36*
PNP	26.3 (5.2)	90.1 (-8.9)	22.6 (-9.6)	98.4 (-1.6)
DBCA	24.6 (-1.6)	93.1 (-6.9)	26.2 (4.8)	101.2 (1.2)
CFCA	27.1 (8.4)	104.1 (4.1)	26.3 (5.2)	90.1 (-9.9)
DCCA*	148.9 (-0.7)	264.8 (-3.8)	24.4 (-2.4)	102.1 (2.1)
TCPY	25.5 (2)	103.2 (3.2)	22.5 (-10.0)	81.2 (-18.8)
3PBA	23.4 (-6.4)	90 (-10)	25.9 (3.6)	76.0 (-24.0)
4F3PBA	20.1 (-19.6)	84.8 (-15.2)	21.9 (-12.4)	65.4 (-34.6)
CDCA	14.8 (-40.8)	94.9 (-5.1)	26.4 (5.6)	100.3 (0.3)
MDA	24.2 (-3.2)	98.2 (-1.8)	25.6 (2.4)	72.0 (-28.0)
Terbufos Sulfone	21.6 (-13.6)	86.7 (-13.3)	24.8 (-0.8)	72.6 (-27.4)
Phorate Sulfone	19.7 (-21.2)	78.9 (-21.1)	24.7 (-1.2)	75.2 (-24.8)
Terbufos Sulfoxide	30.6 (22.4)	91.5 (-8.5)	27.2 (8.8)	73.6 (-26.4)
Phorate Sulfoxide	27.2 (8.8)	104.7 (4.7)	25.4 (1.6)	72.6 (-27.4)
IMPY	25.7 (2.8)	108.1 (8.1)	25.4 (1.6)	71.0 (-29.0)

Table 3.19 Accuracy Comparison between MISPE and Ion-Exchange SPE.

The trend observed for the accuracy measurements for both methods leads to one possible conclusion. As mentioned in the introduction and again in the ion-exchange method chapter, perhaps some of the target analytes are bound tighter to the soil over time, thus limiting the extractable amount. QC pools are mixed and air-dried for 96 hours before the first analysis. In contrast, standard curve materials are spiked and immediately processed. This problem could be mitigated if standard curve materials were spiked ahead of time and stored until analysis and thus the extractable amounts would most likely mimic those found in the QC materials. However, this trend is not reflected with the QCL materials with the MISPE method. Therefore, it is concluded that the fortification scheme needs to be re-evaluated to improve accurate response.

Linearity

Similar correlation coefficients were found with the MISPE method, shown in Table 3.20, as compared with the ion-exchange method. All target analytes had R^2 values ≥0.99 which showed that the spiked standard curve showed excellent linear results.

Linearity	
Analyte	R^2
PNP	0.999
DBCA	0.999
CFCA	0.994
DCCA	0.998
TCPY	0.996
3PBA	0.998
4F3PBA	0.999
CDCA	0.995
MDA	0.999
Terbufos Sulfone	0.996
Phorate Sulfone	0.991
Terbufos Sulfoxide	0.996
Phorate Sulfoxide	0.994
IMPY	0.989

Table 3.20 Linearity of Target Analytes with MISPE.

Ion Suppression/Ion Enhancement

As stated above, matrix-related effects such as ion suppression/enhancement can lead to sub-optimal method validation parameters that decrease the method's overall reliability. Each target analyte was assessed for suppression/enhancement effects using the MIPSE method to compare with adverse effects seen with the ion-exchange method. Post-extract samples were spiked with standard and compared with solvent standards as was performed with the ion-exchange method.

MDA

The warped peak for MDA resulting from some unknown matrix effect with the ion-exchange method was reduced almost completely with the MISPE method as shown below in Figure 3.55. However, the signal is still slightly suppressed with slightly less intensity seen with the post-extract sample as compared with the solvent sample.

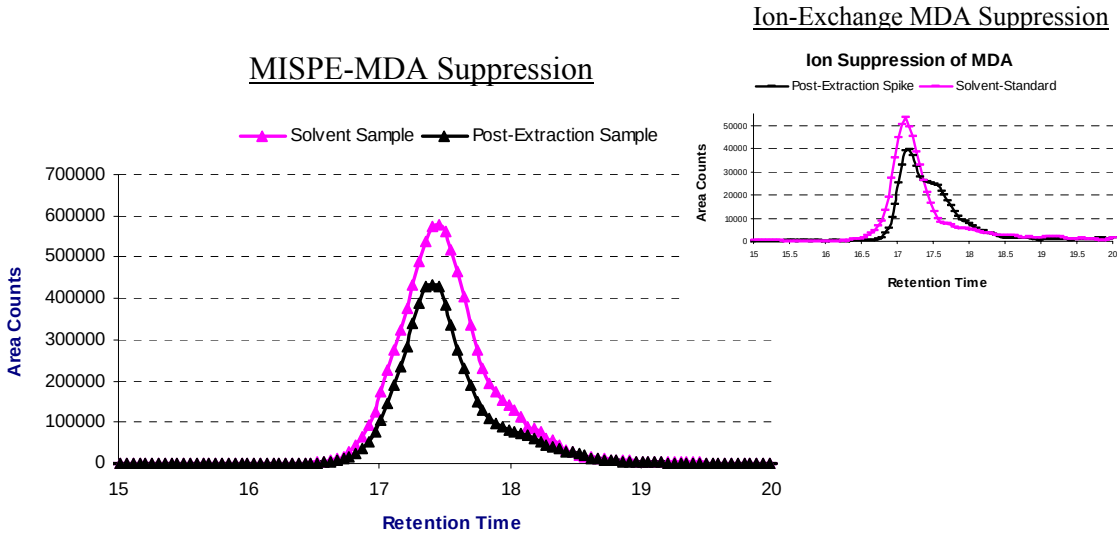


Figure 3.55 MDA Ion Suppression Comparison between MISPE and Ion-Exchange SPE.

CFCA

With ion-exchange, CFCA was suppressed completely with one parent-daughter ion pair (*m/z* 241-121) and less suppressed with another parent-daughter ion pair (*m/z* 241-205). The opposite effect occurred with the MISPE method as shown in Figure 3.56. CFCA was enhanced by approximately 4x the signal as that seen with the solvent sample (for both parent-daughter pairs). This would indicate that perhaps some unknown interference causing the enhancement results from the MISPE system and not the matrix as was first believed because the only variable that changed was the SPE technique (all samples came from the same soil pool).

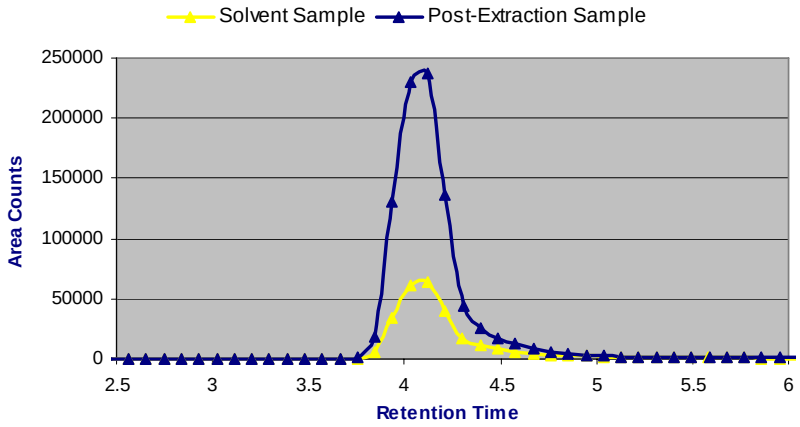


Figure 3.56 Ion Enhancement of CFCA with MISPE.

Overall, all target analytes (except CFCA) did not exhibit any significant suppression or enhancement effects associated with the MISPE method.

Conclusions

Non-specific DAPs were excluded from MISPE analysis due to non-recoverable analytes (all DAPs broke thru MIP cartridges in either the loading or wash step(s)). Therefore, only pyrethroid and specific OPs degradation products were analyzed using MISPE and compared with the ion-exchange method. Overall, the MISPE method yielded detection limits in the high pptr-low ppb range (0.66-9.84ng/g) for all target analytes. Analyte recoveries were high; all target analytes yielded recoveries between 78%-102% excluding IMPY which had an extremely low recovery of 4%. Overall precision measurements (RSD) at QCL and QCH levels were between 9.1-21.4% and 6.6-14.9% respectively. This would indicate that both the lower and higher end of the calibration curve could be useful for quantitative purposes (except for CDCA, CFCA and IMPY at the lower end). The reduction in matrix effects problems (excluding CFCA) was observed with MIPSE results which also seemed to improve method robustness (there was no maintenance required during all the MISPE method validation runs ~ 500 samples).

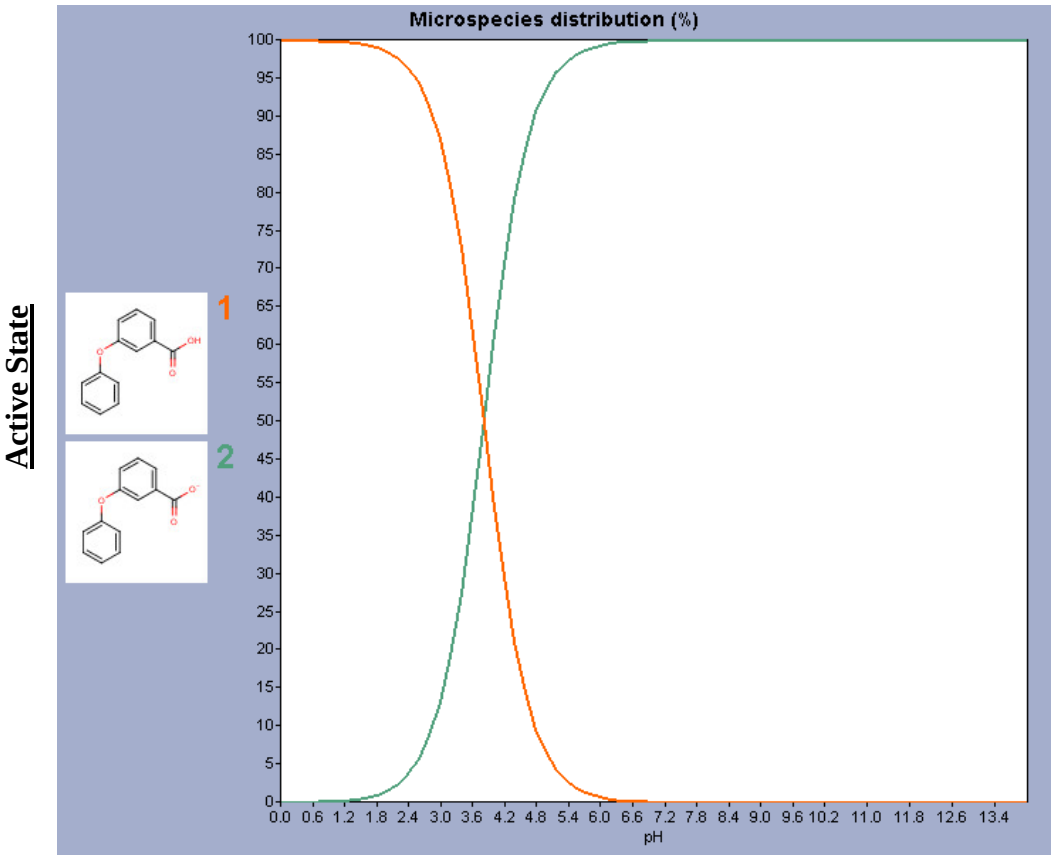
The MISPE method clearly shows advantages over the ion-exchange method with lower detection limits and more precise measurements allowing quantification of all target analytes at the higher end and most at the lower end of the calibration curve. Therefore, the advantage of utilizing the highly selective MIP sorbent is shown in comparison with the more conventional ion-exchange sorbent. Another distinction between the two SPE methods is method robustness. All method development and validation assays for MISPE analysis were run on the instrument (LC-Quantum) without any apparent decrease in sensitivity or any maintenance performed (~1000 samples). Therefore, the

MISPE method must decrease matrix interferences that are not removed with the ion-exchange method (that required routine maintenance). The one disadvantage is that with higher selectivity, the more exclusive the method becomes-ergo the exclusion of DAPs. However, for quantitative analysis of pyrethroid and specific OP degradation products, the MISPE method is favored over the ion-exchange method.

Appendix 3A

The following figures detail physiochemical details of target analytes (generated from ChemAxon© MarvinSketch version 1.4.6):

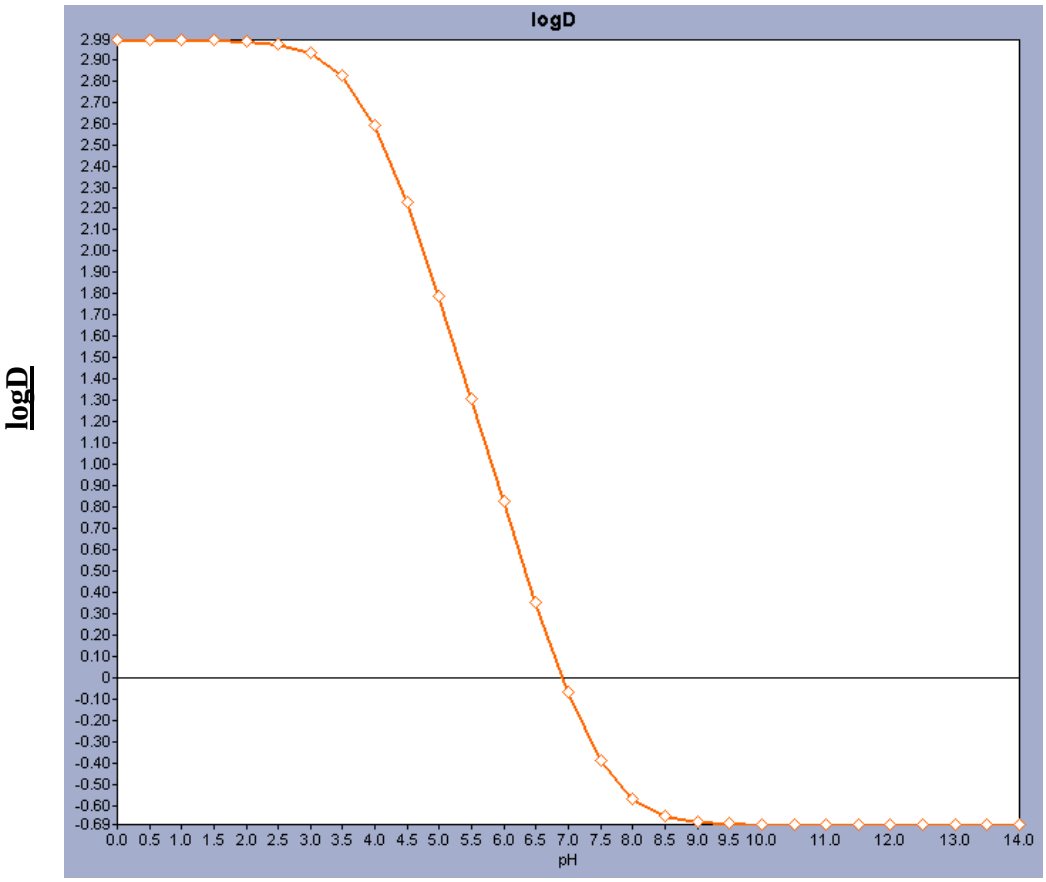
1. 3-PBA pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

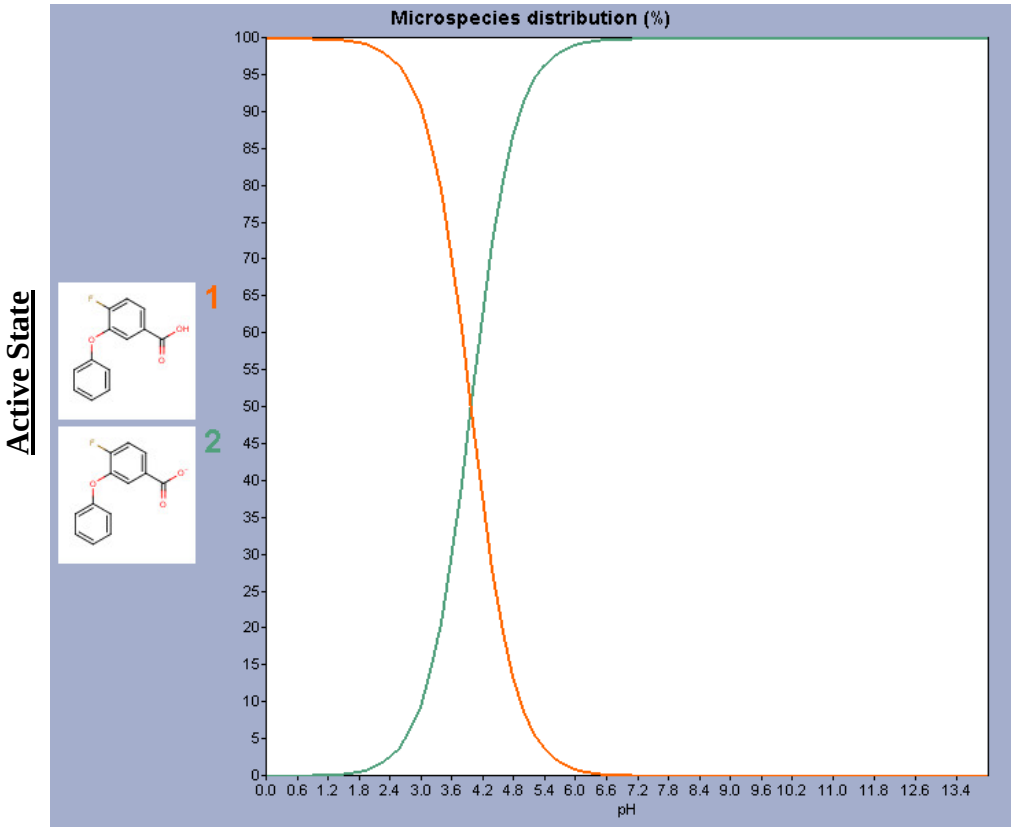
3-PBA – logD Determination



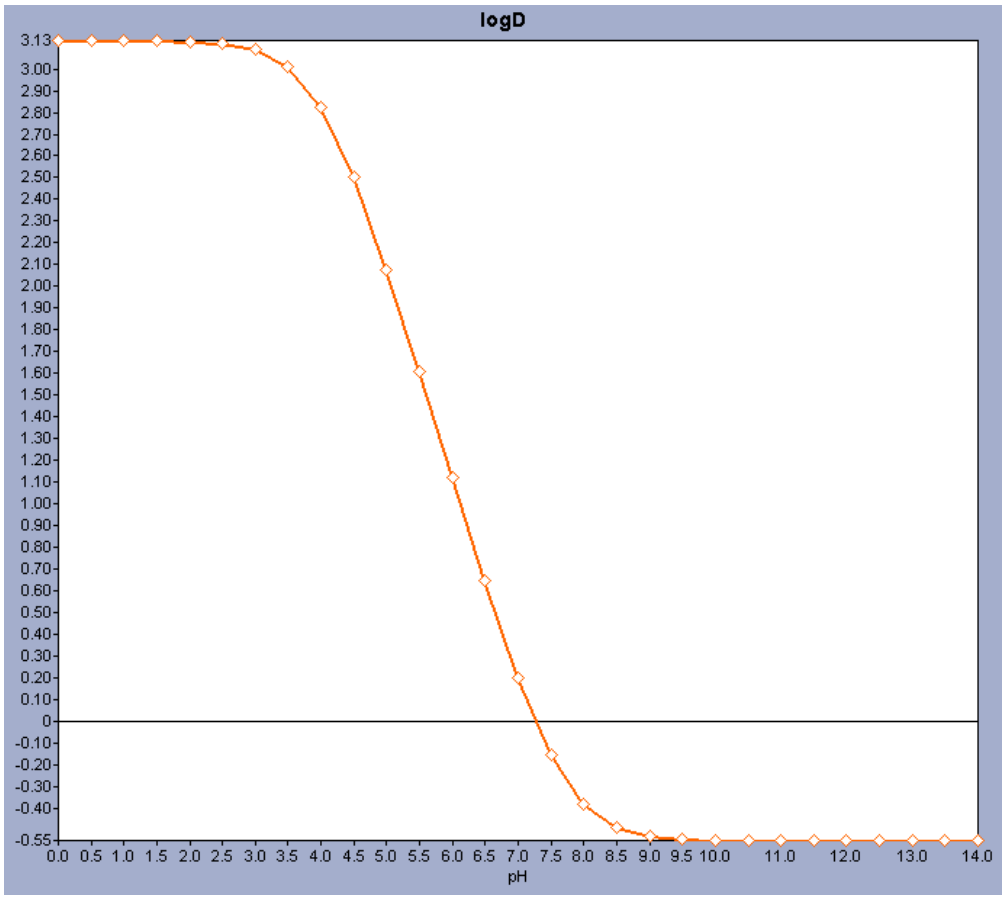
logD

pH

2. 4F3-PBA pKa Determination – 1 ionizable atom between pH1-14



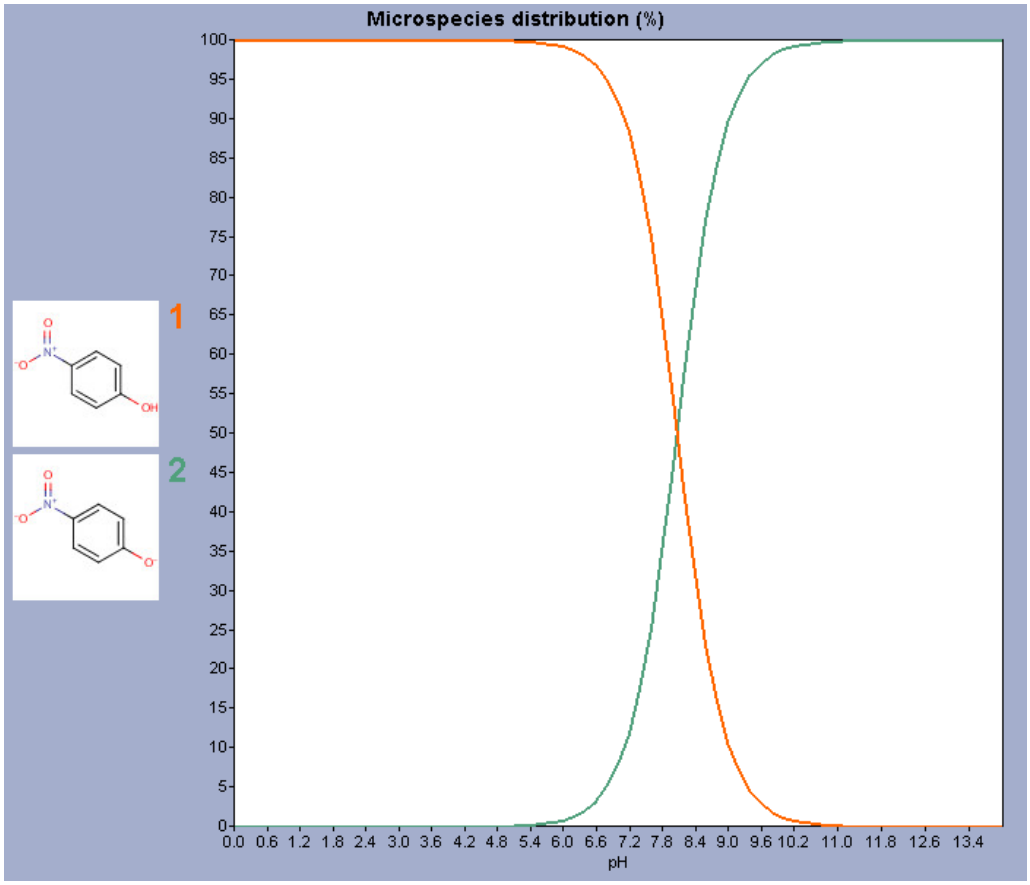
4F3-PBA – logD Determination



logD

pH

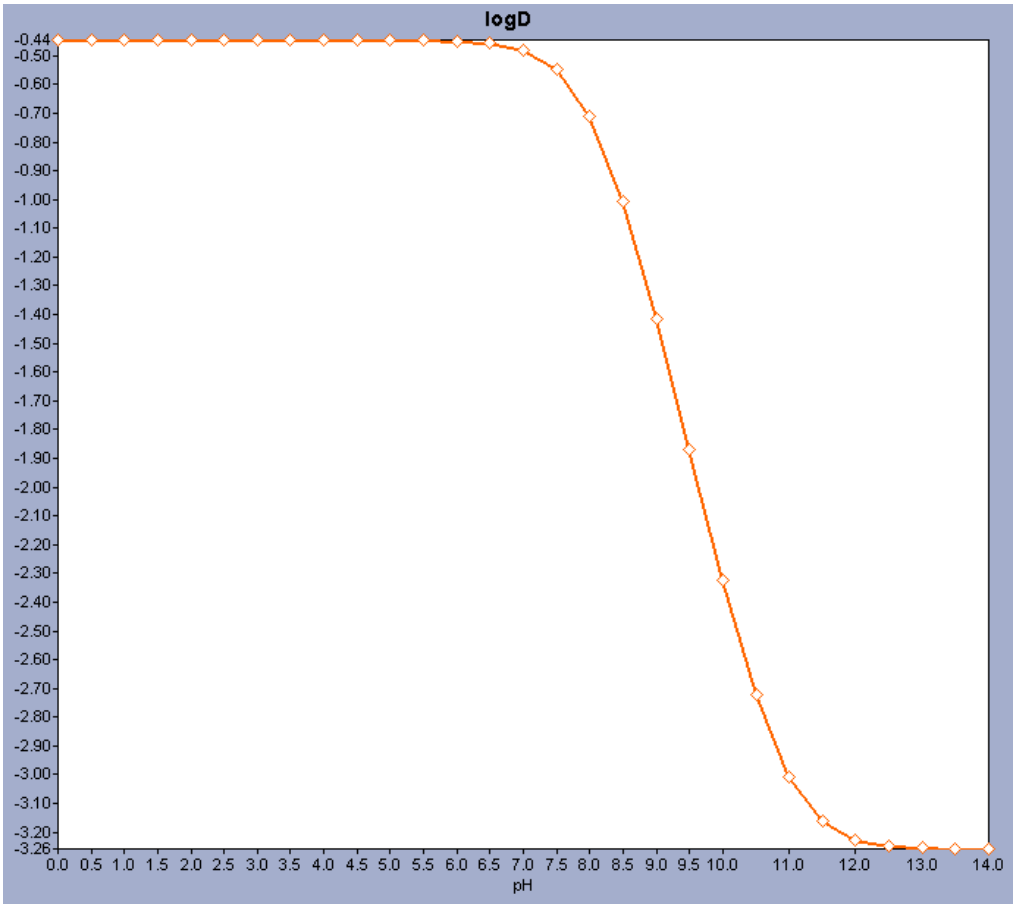
3. 4-Nitrophenol pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

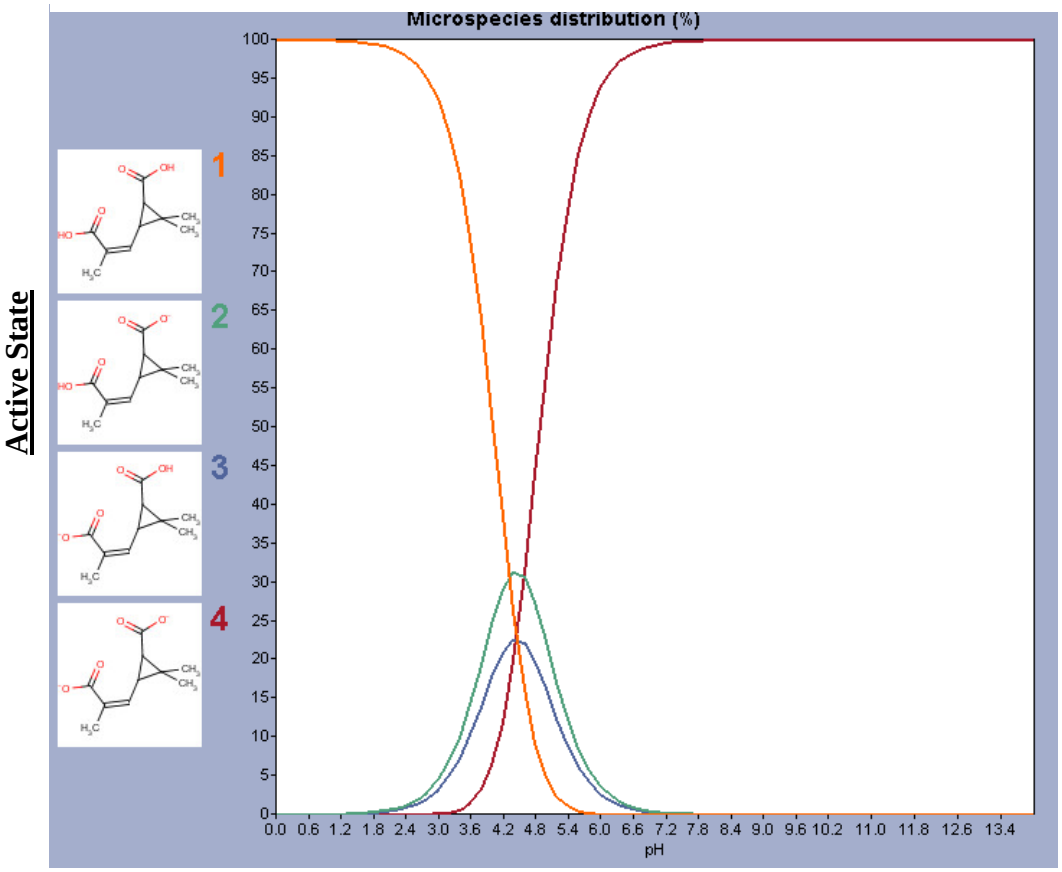
4-Nitrophenol – logD Determination



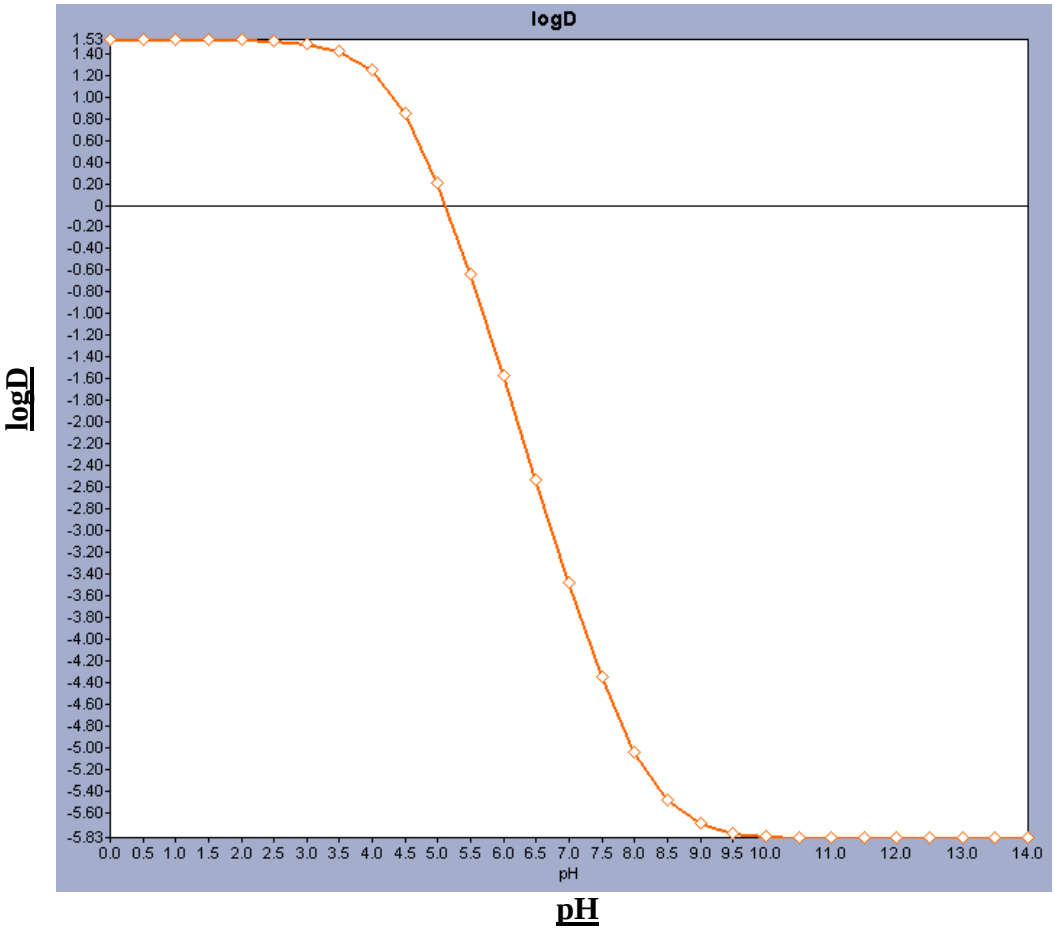
logD

pH

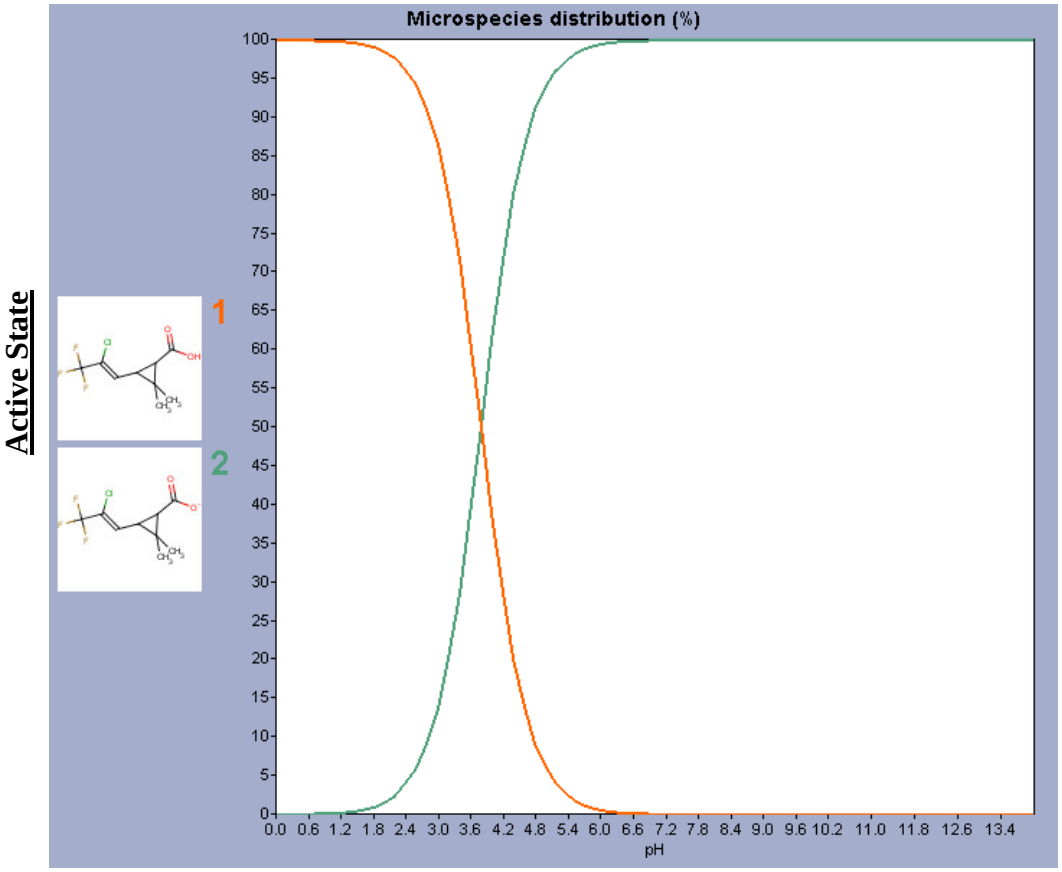
4. CDCA pKa Determination – 2 ionizable atoms between pH1-14



CDCA – logD Determination



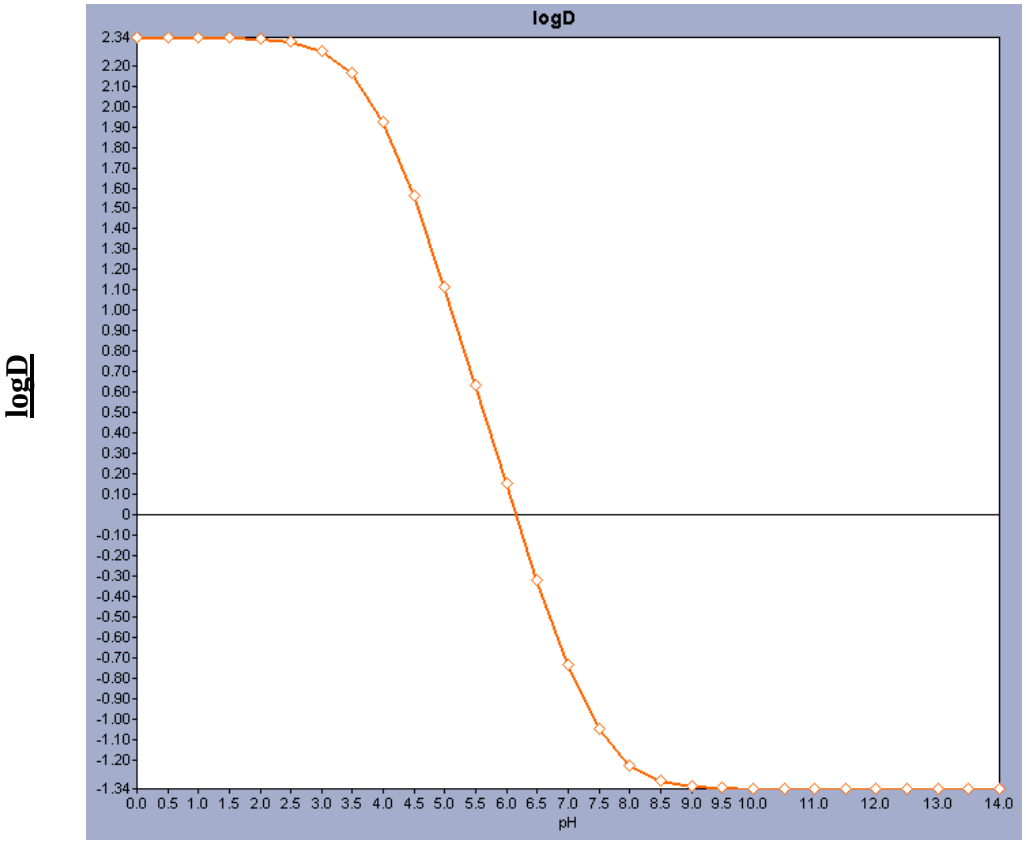
5. CFCA pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

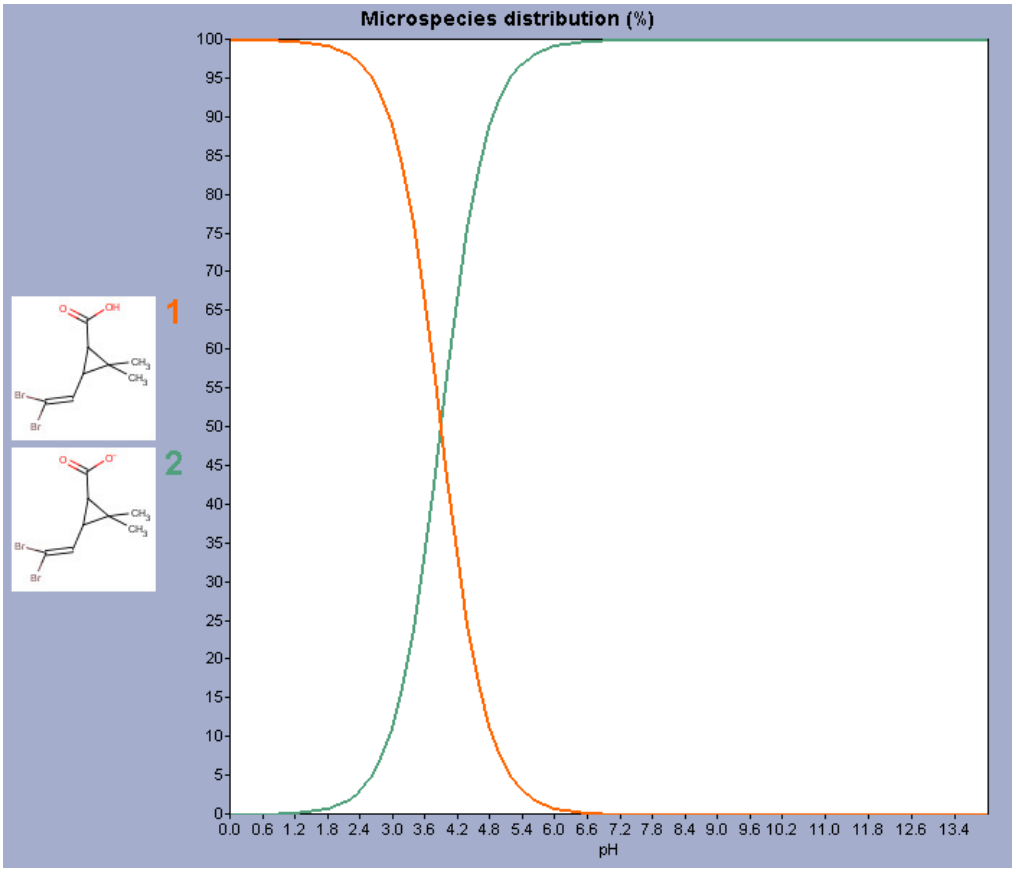
CFCA – logD Determination



logD

pH

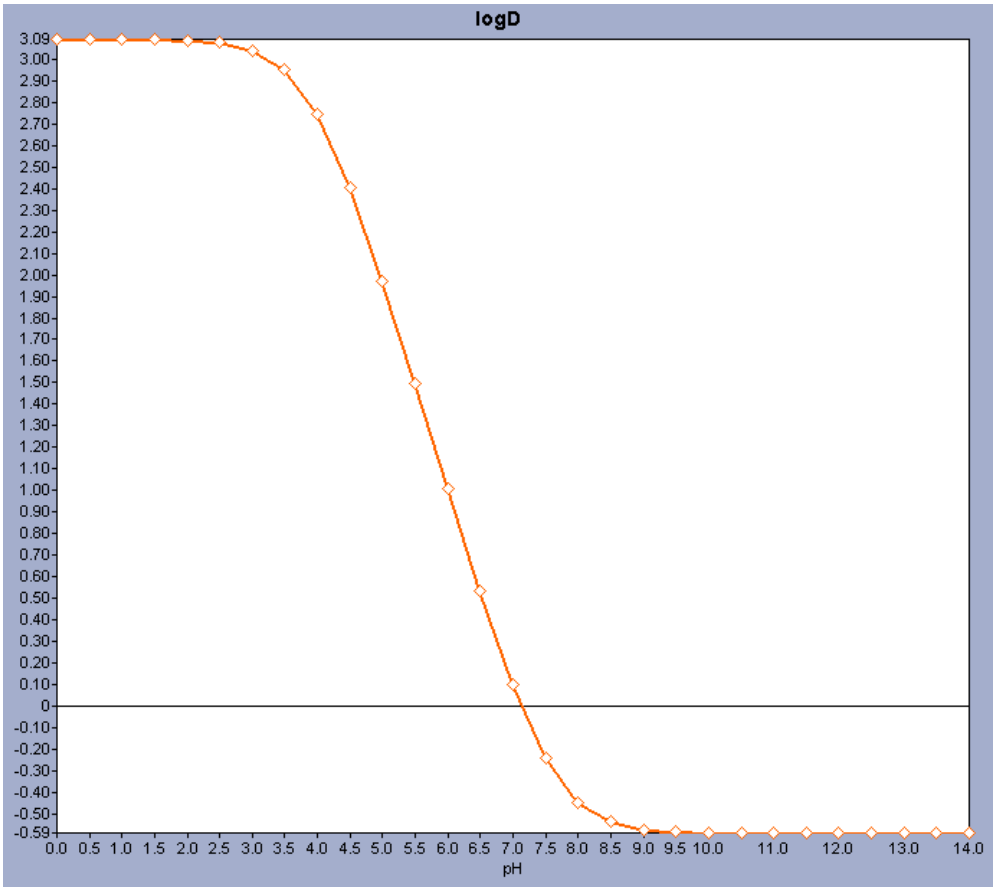
6. DBCA pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

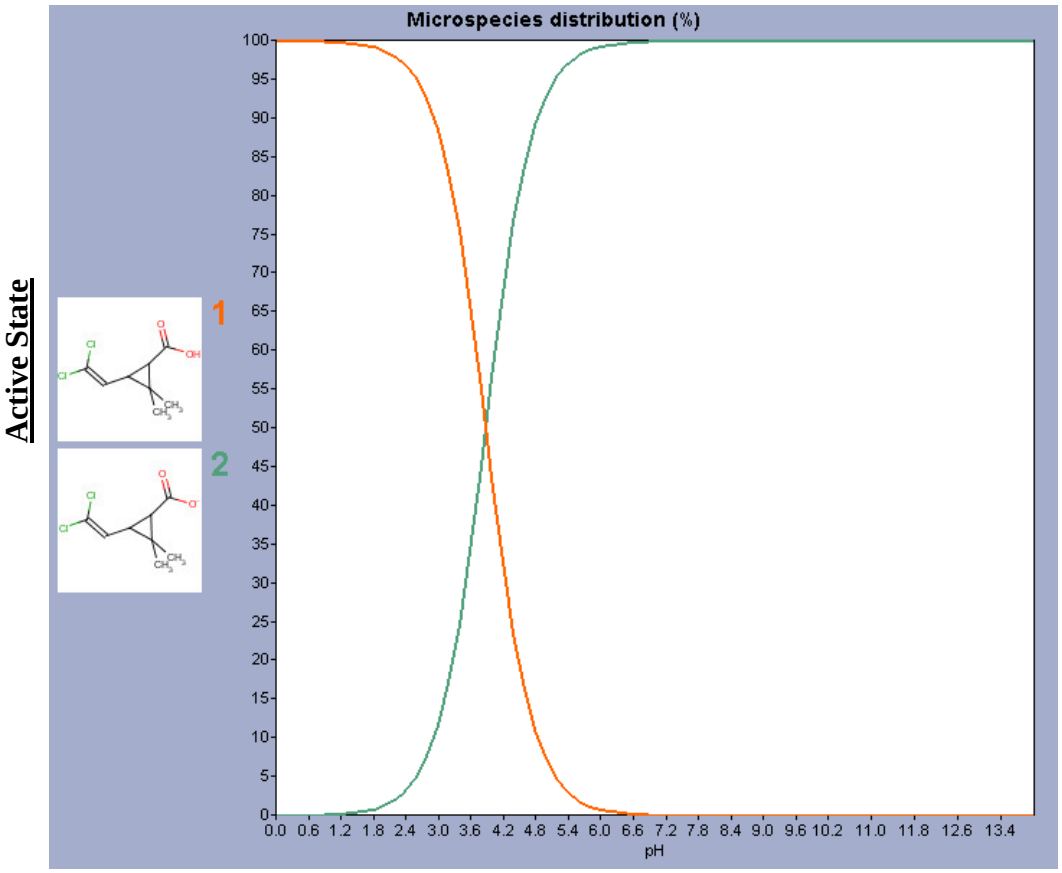
DBCA – logD Determination



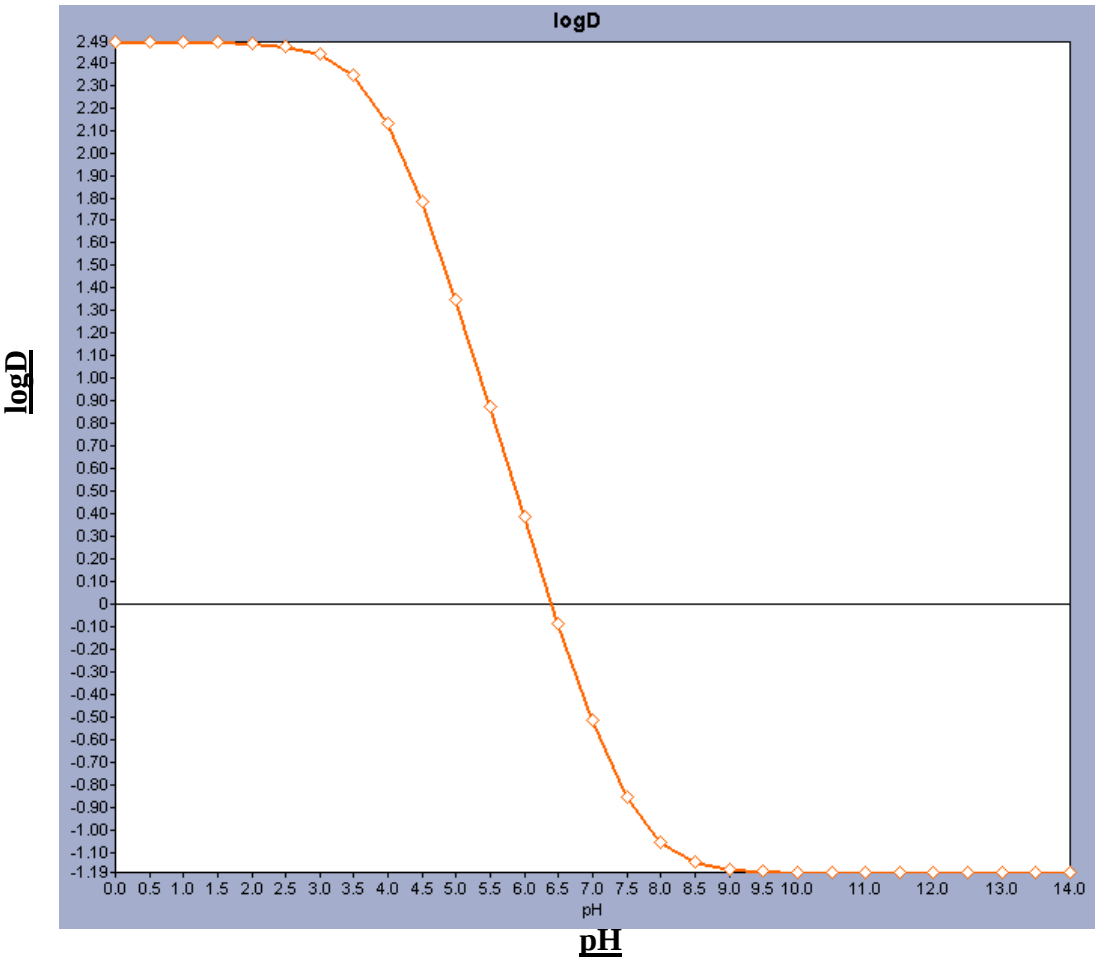
logD

pH

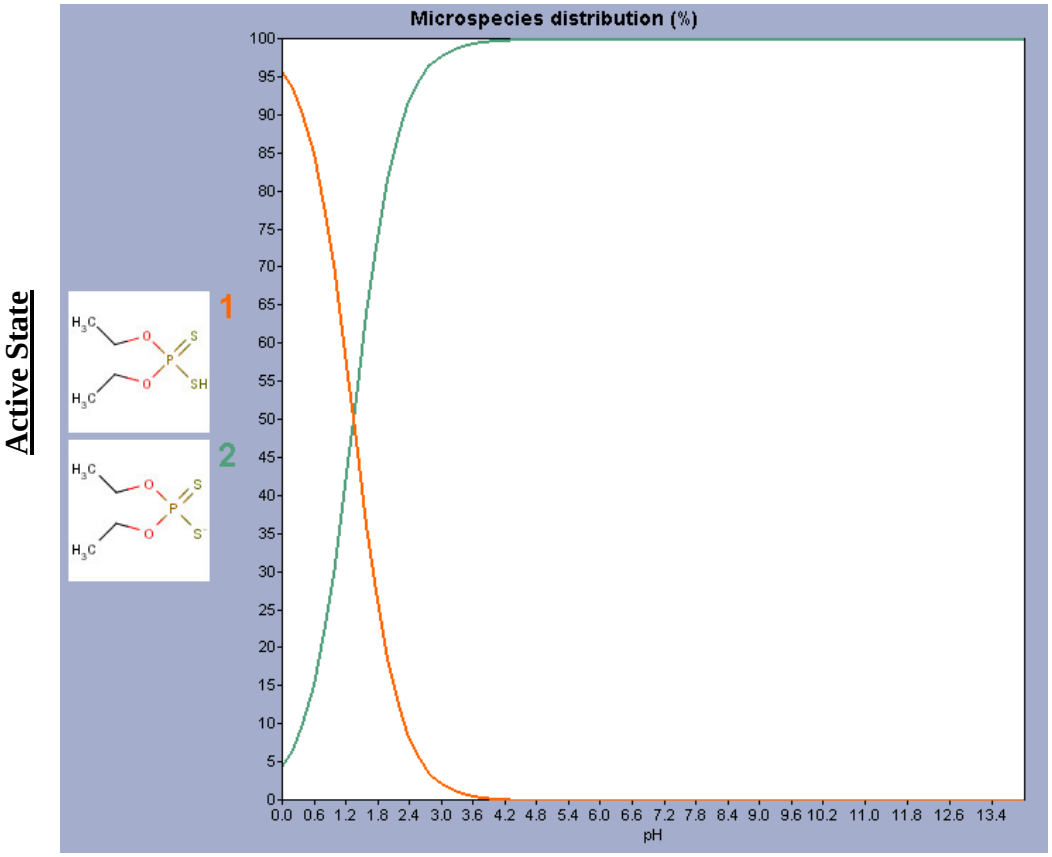
7. DCCA pKa Determination – 1 ionizable atom between pH1-14



DCCA – logD Determination



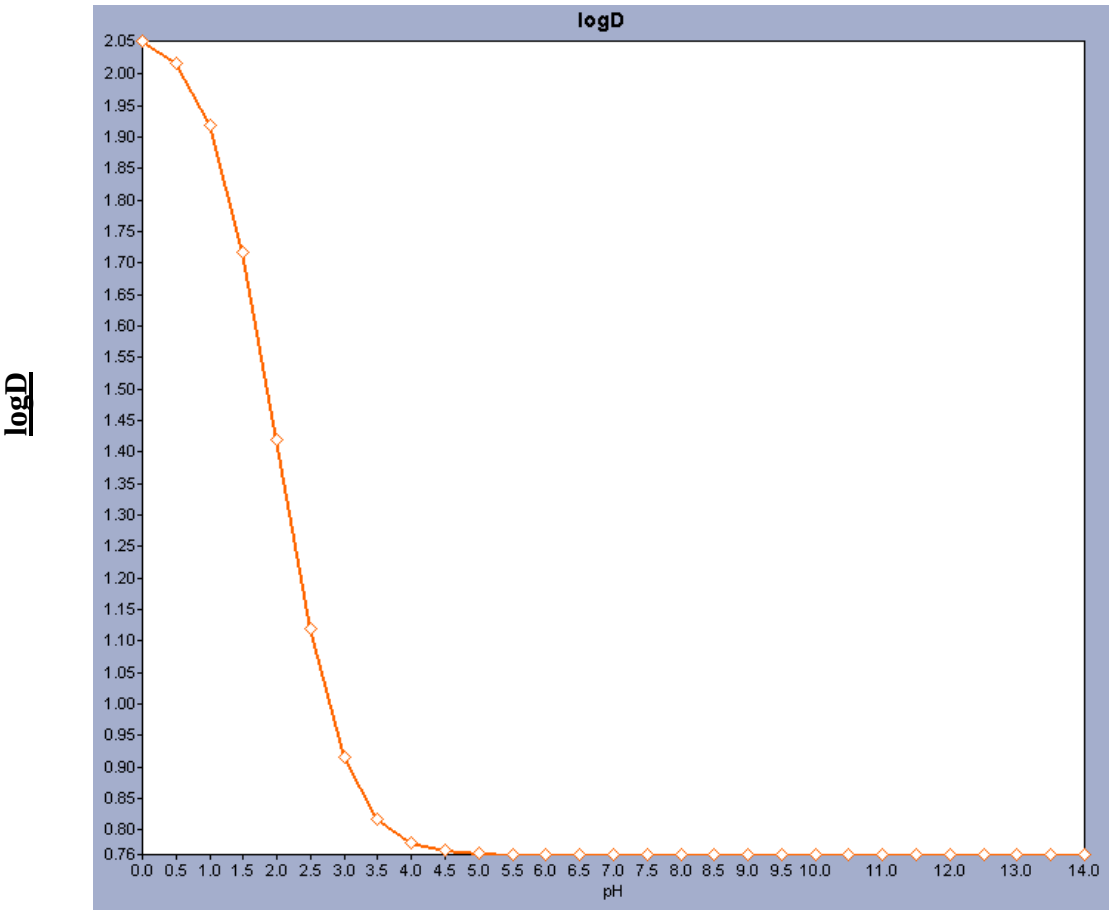
8. DEDTP pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

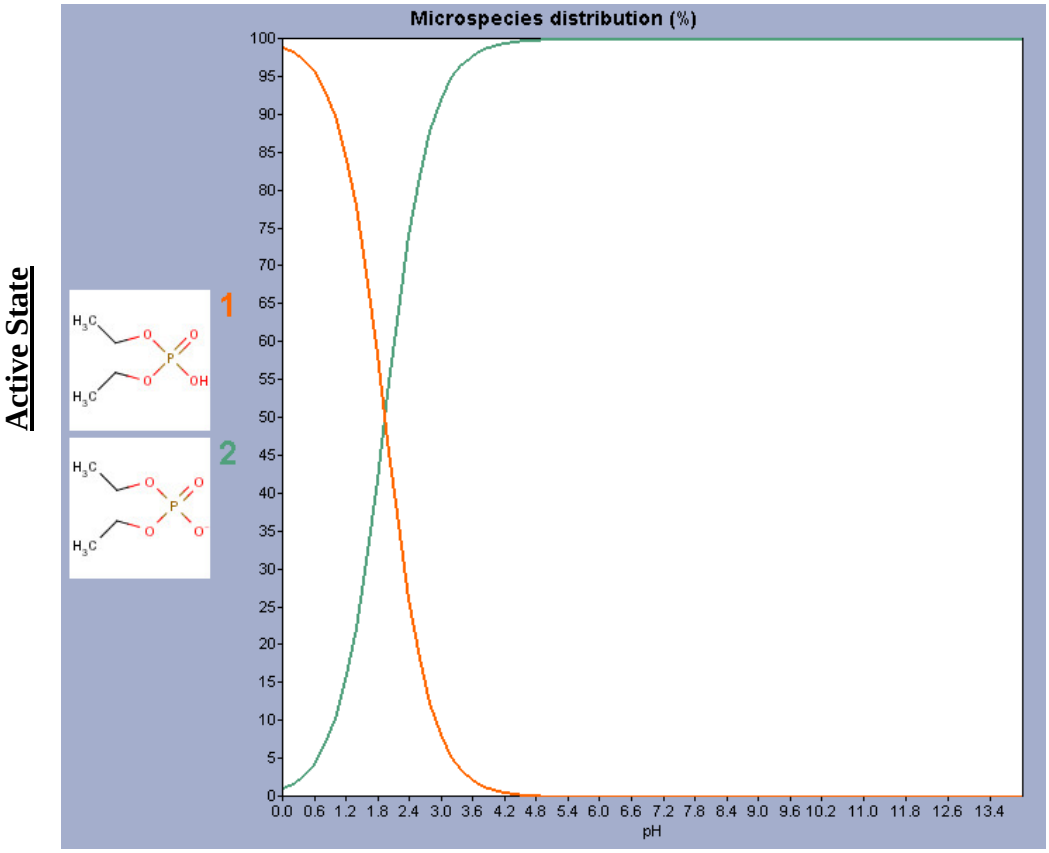
DEDTP – logD Determination



logD

pH

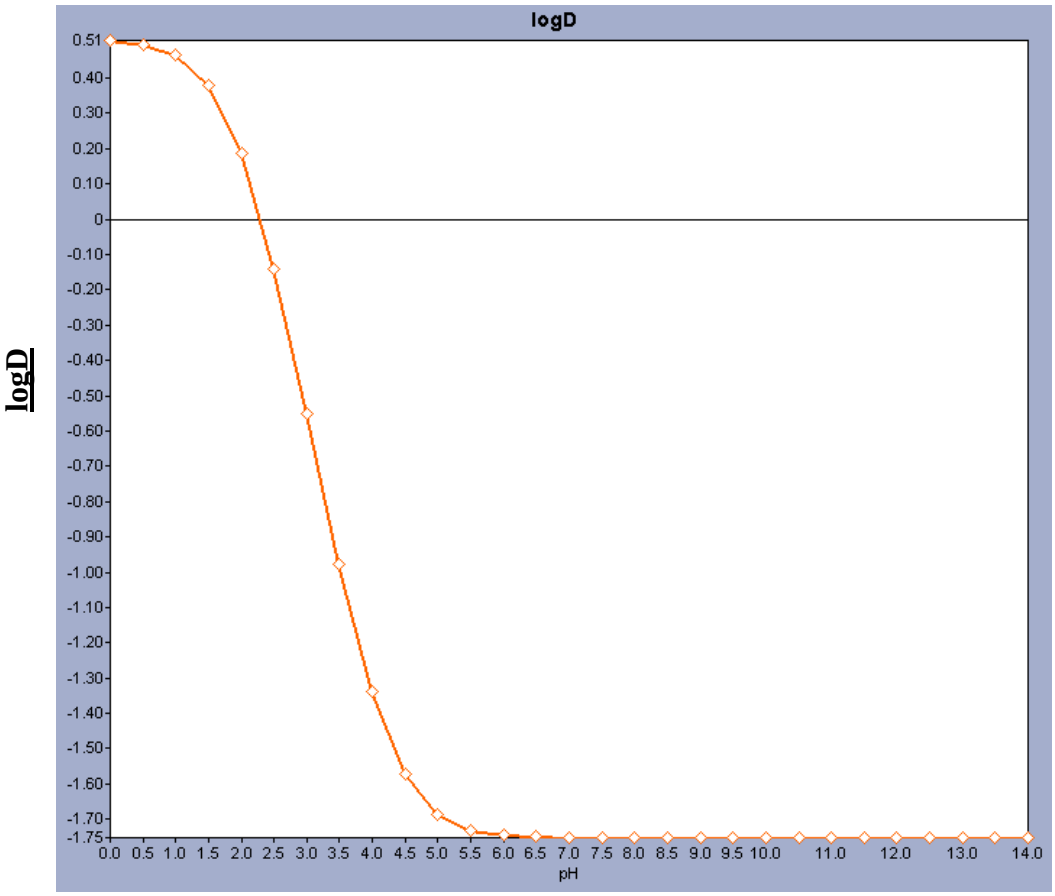
9. DEP pKa Determination – 1 ionizable atom between pH1-14



Active State

pH

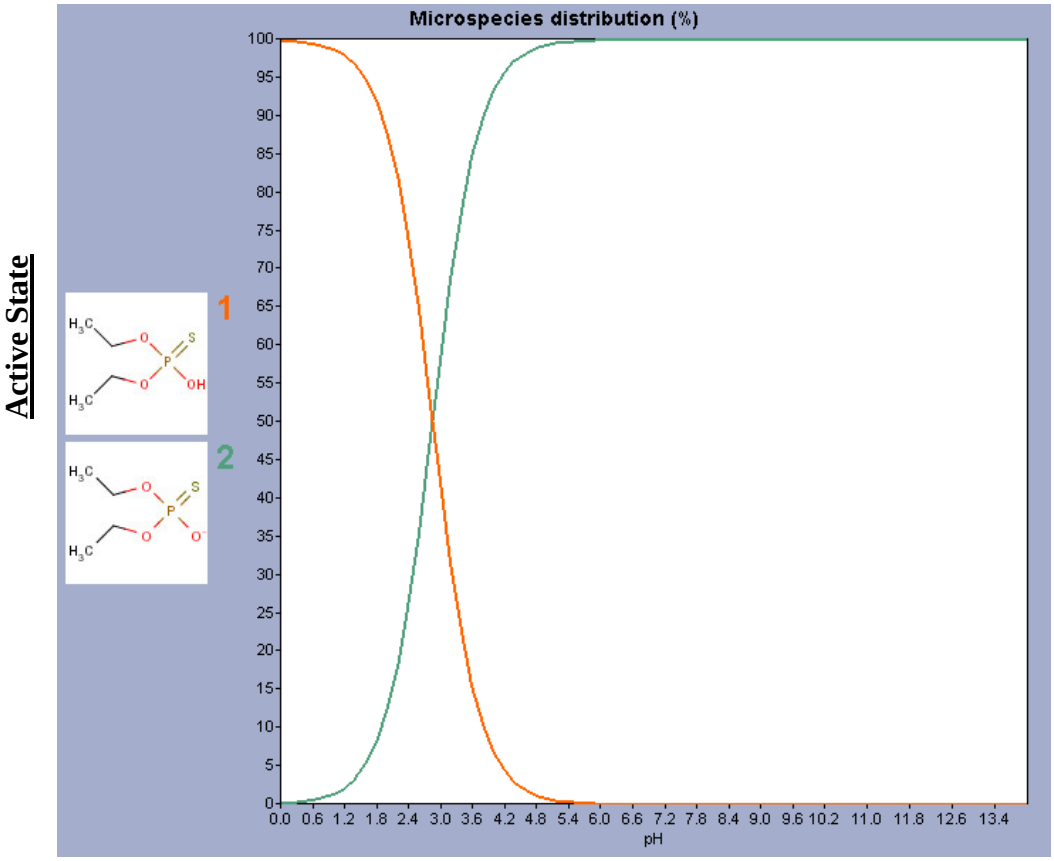
DEP – logD Determination



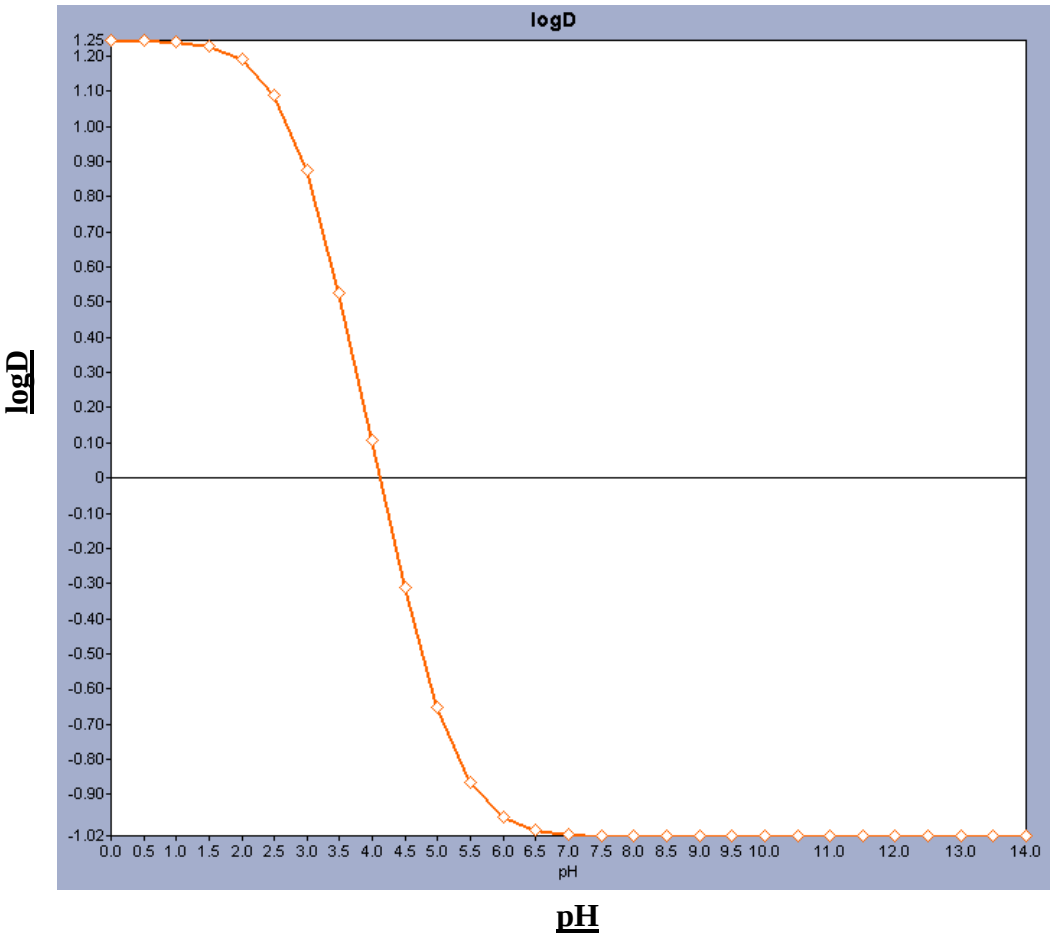
logD

pH

10. DETP pKa Determination – 1 ionizable atom between pH1-14



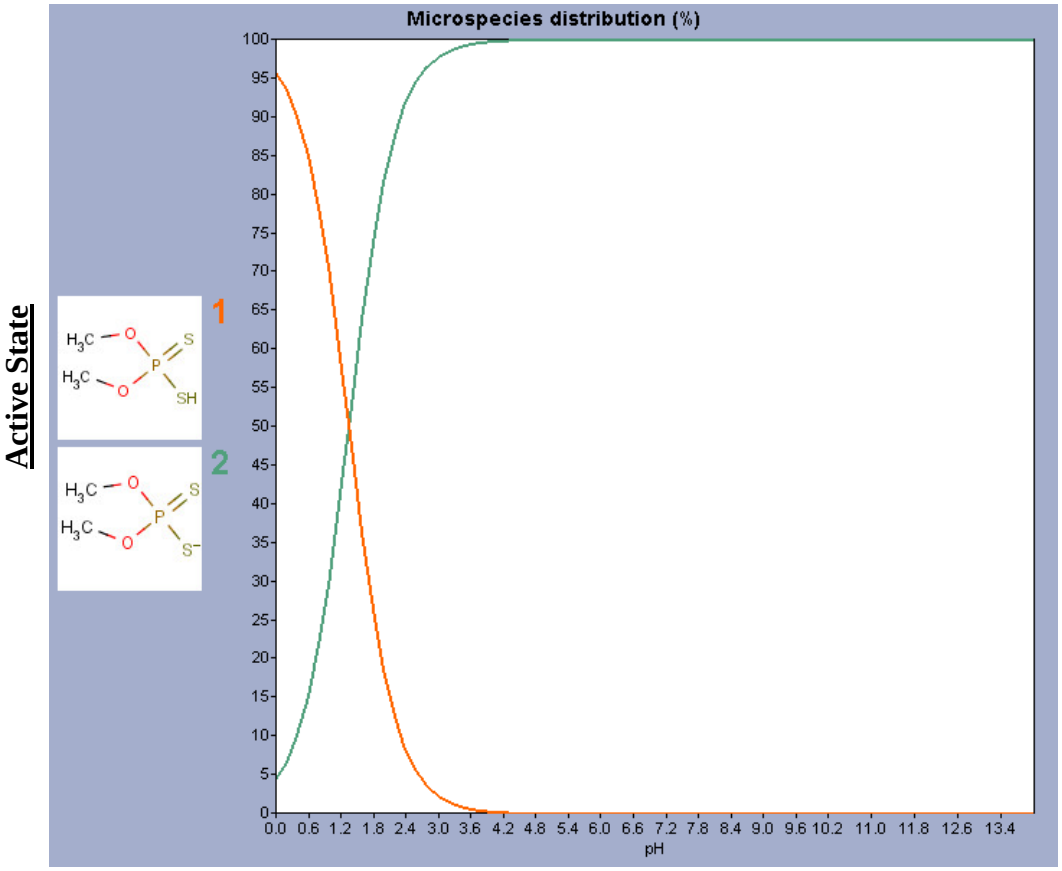
DETP – logD Determination



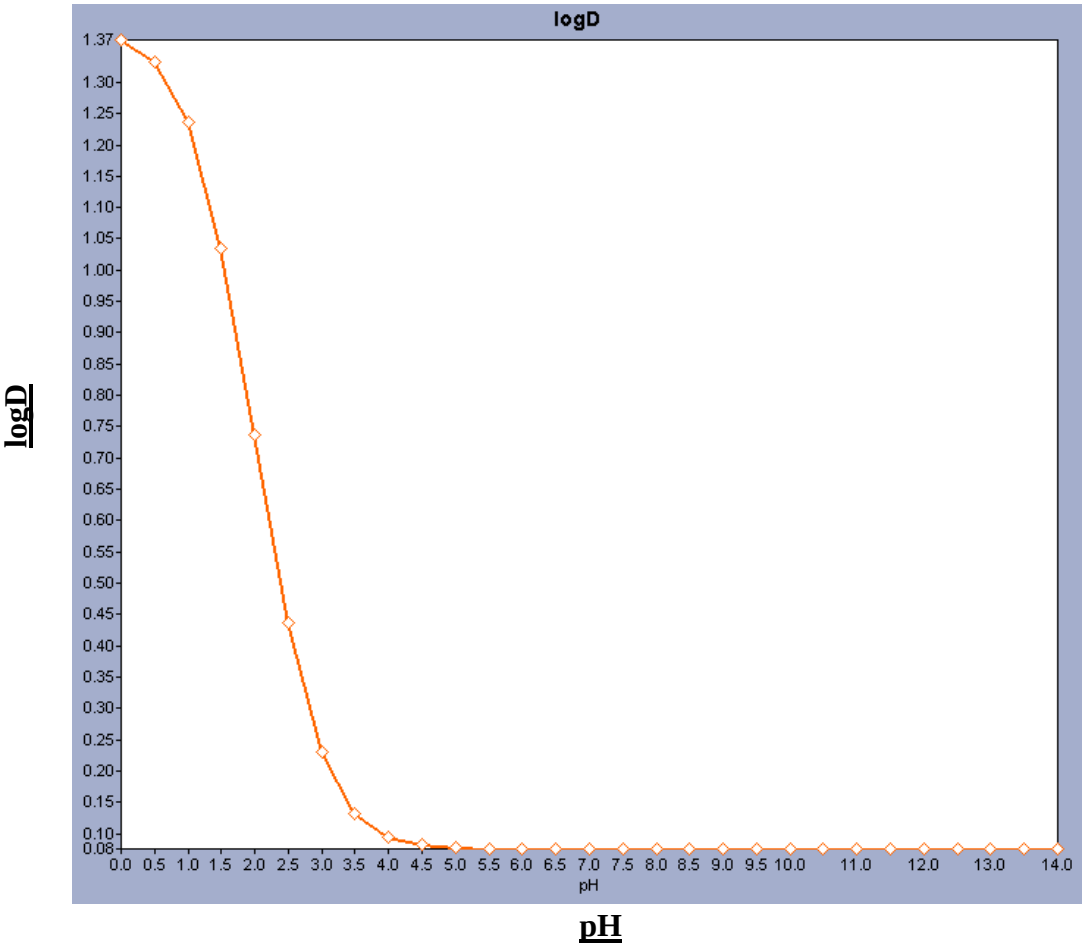
logD

pH

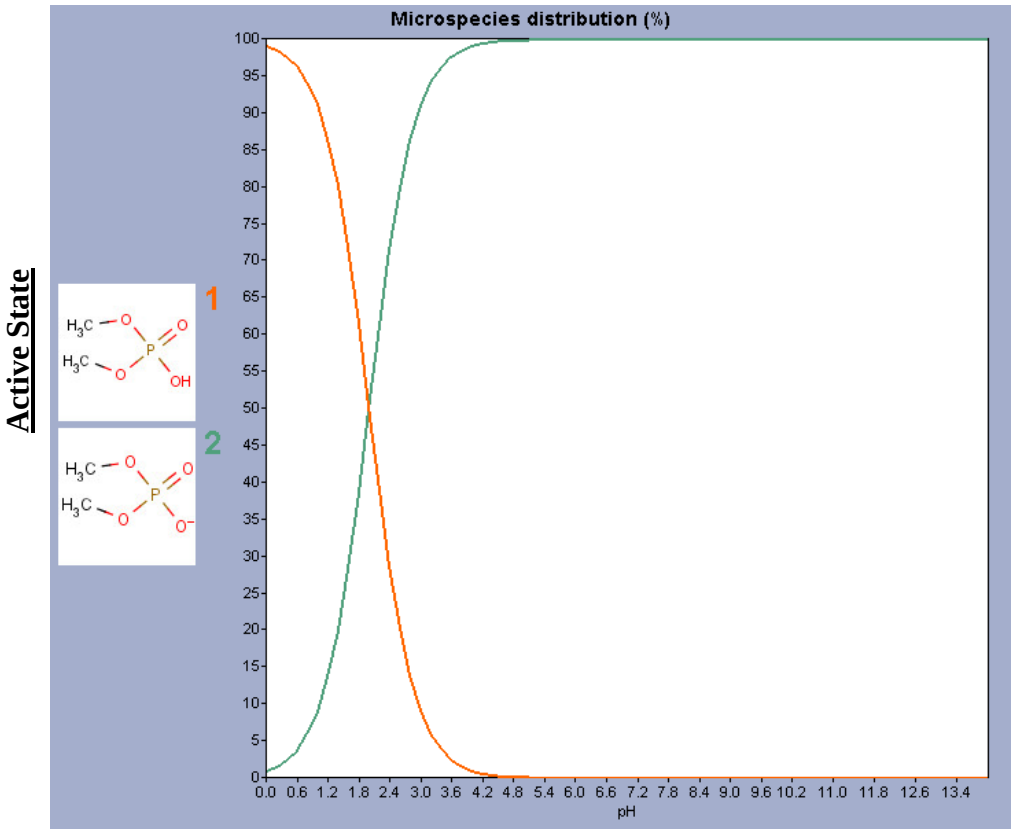
11. DMDTP pKa Determination – 1 ionizable atom between pH1-14



DMDTP – logD Determination



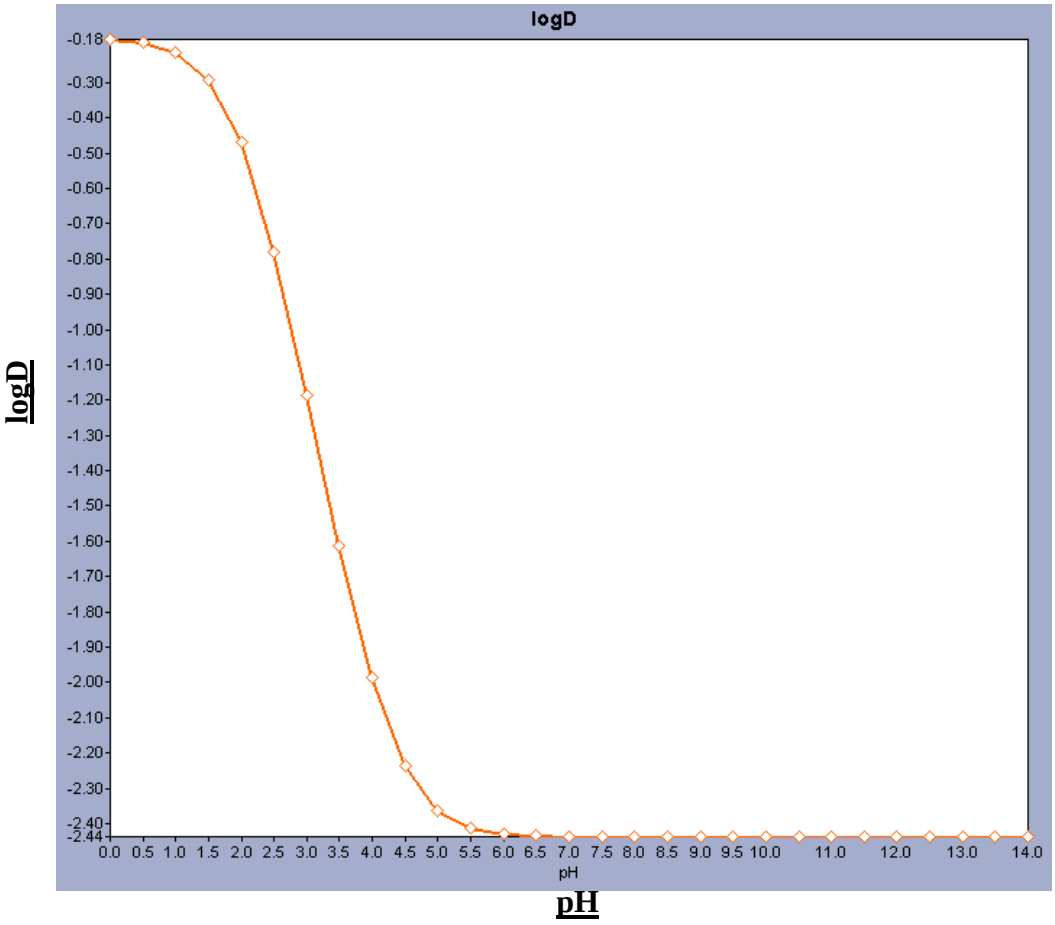
12. DMP pKa Determination – 1 ionizable atom between pH1-14



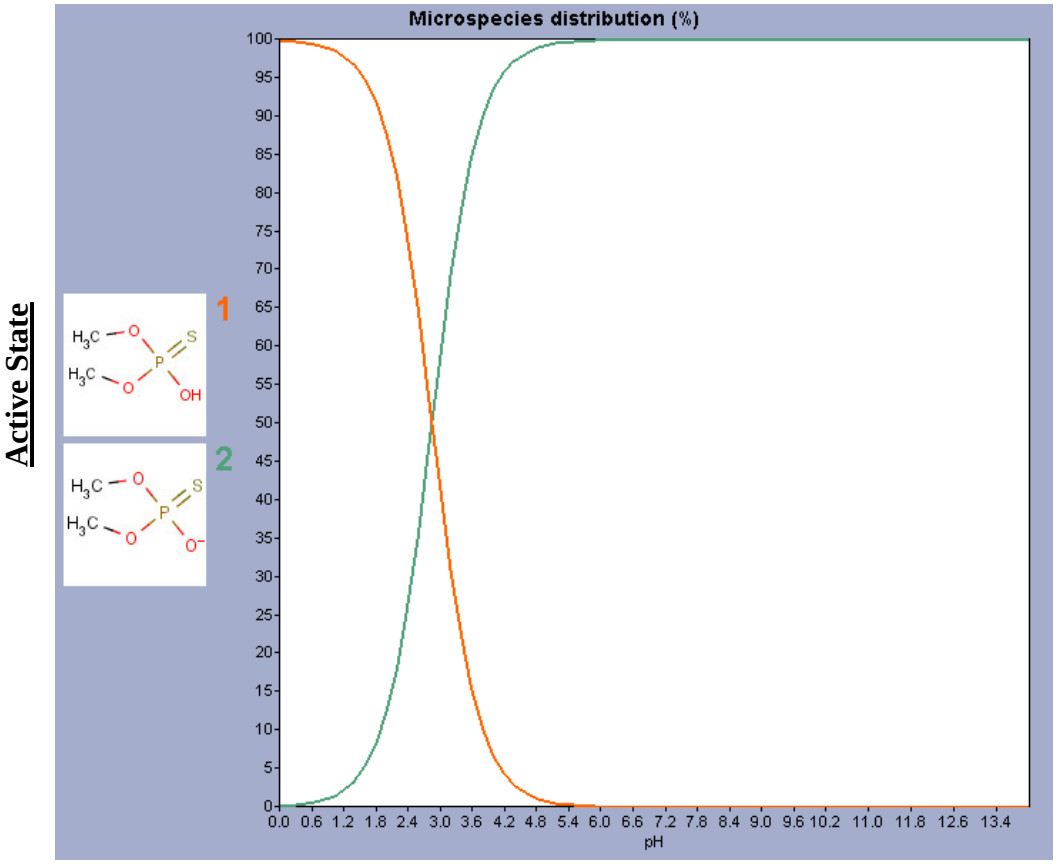
Active State

pH

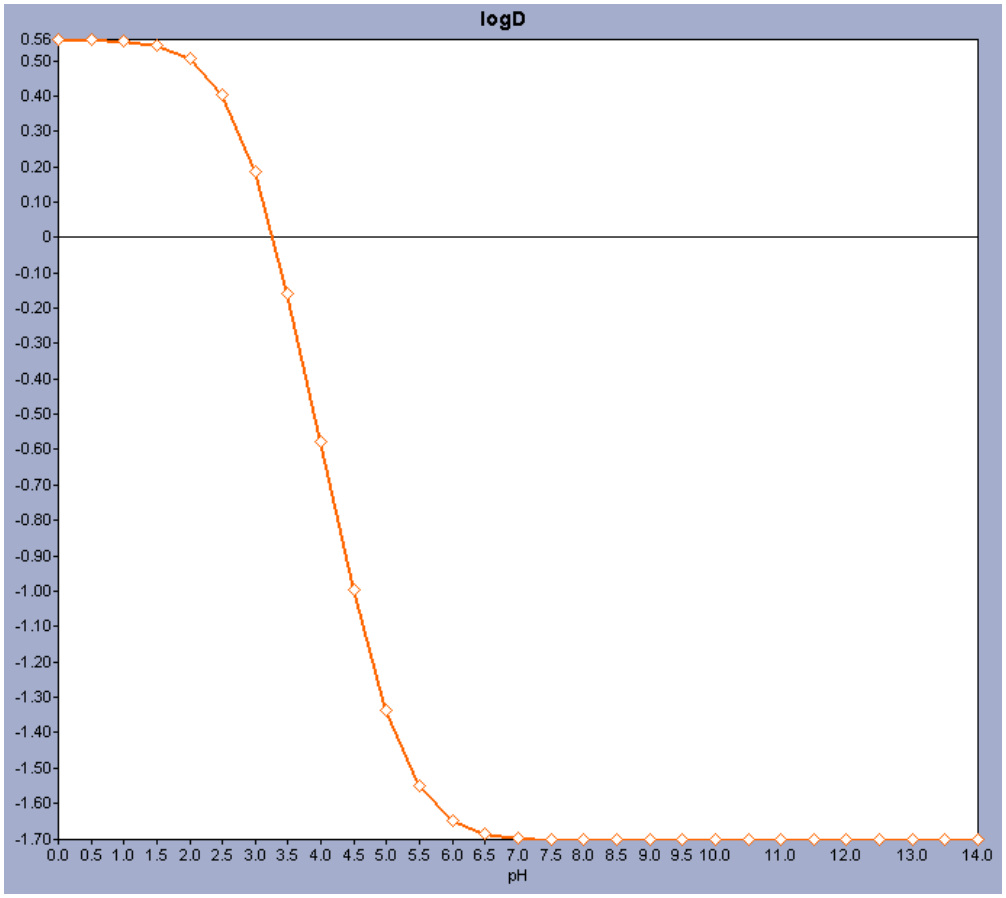
DMP – logD Determination



13. DMTP pKa Determination – 1 ionizable atom between pH1-14



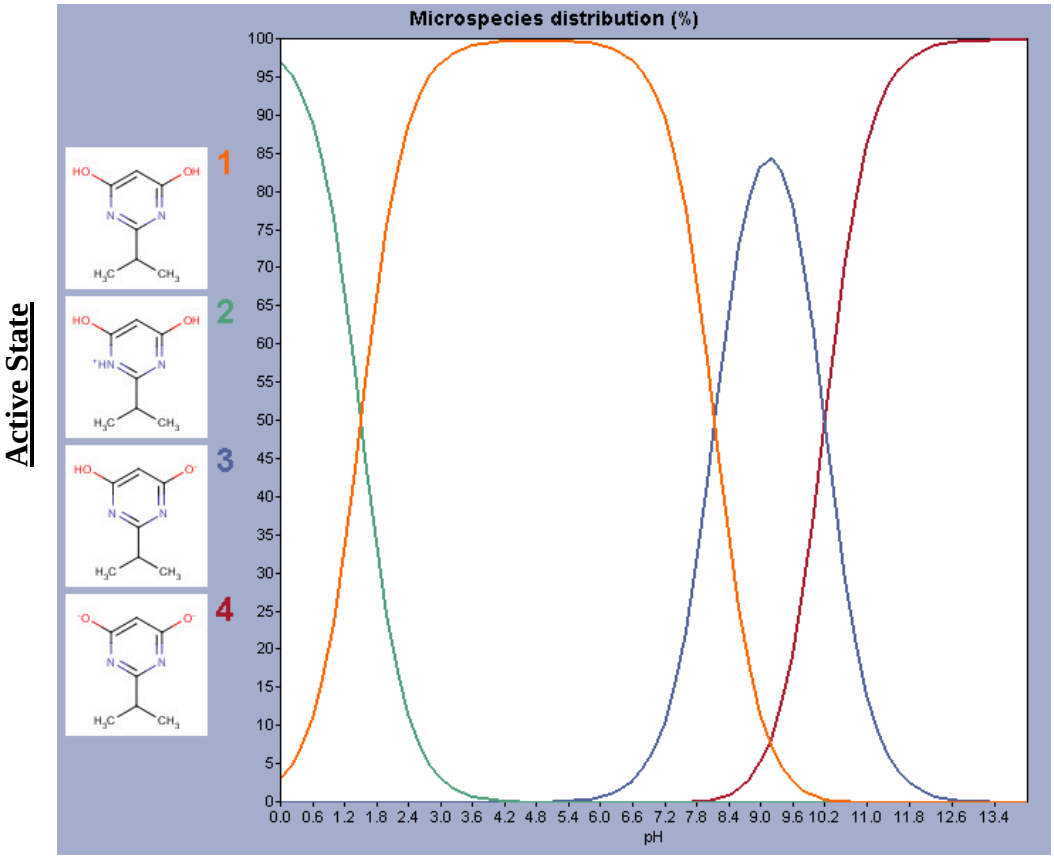
DMTP – logD Determination



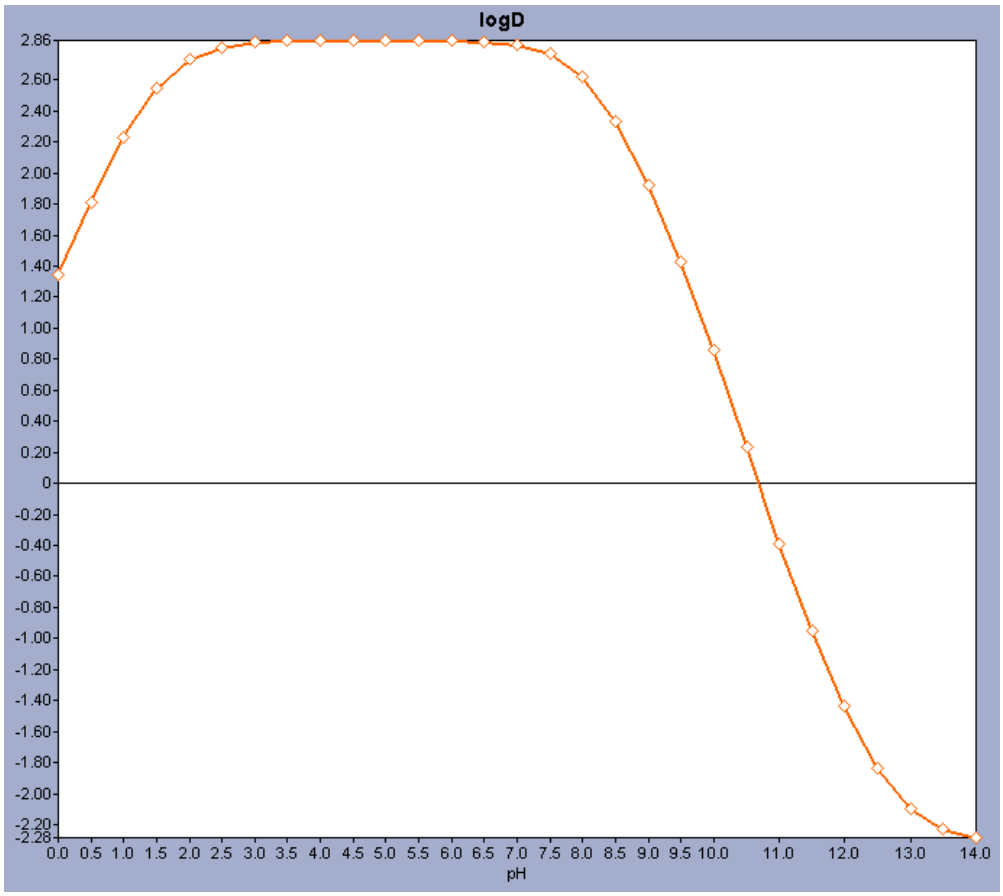
logD

pH

14. IMPY pKa Determination – 3 ionizable atoms between pH1-14



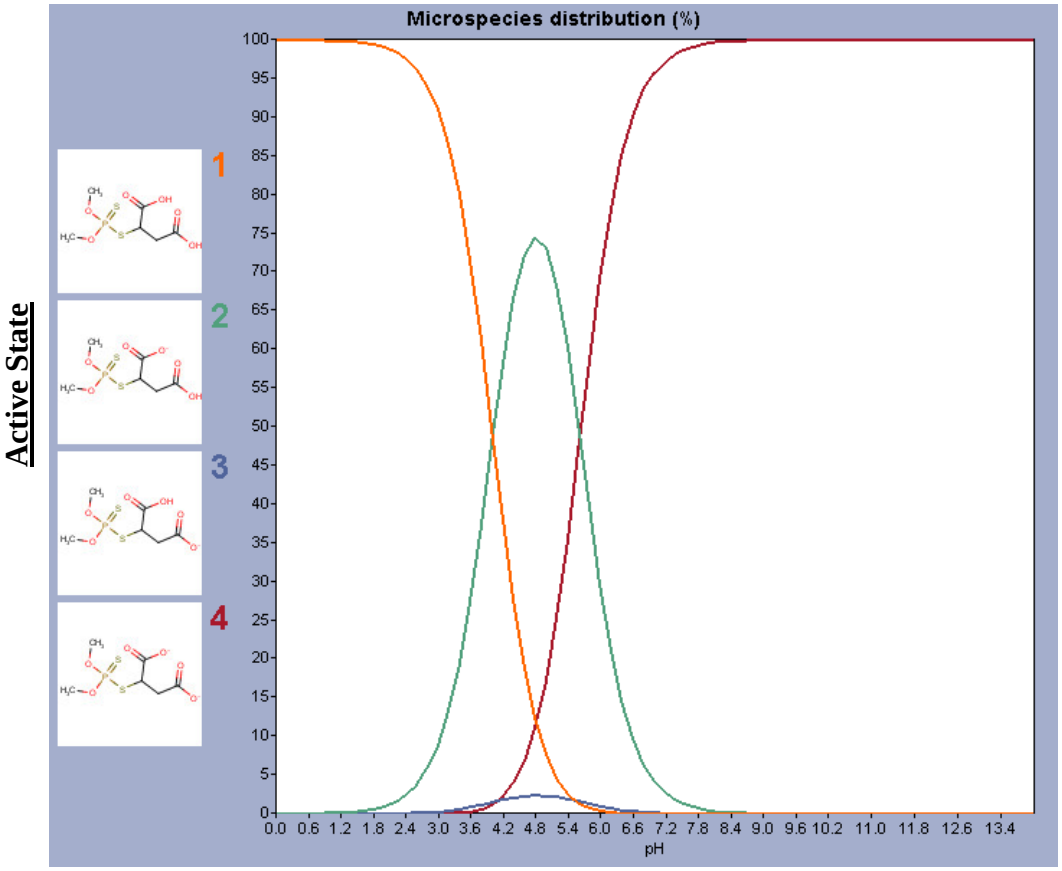
IMPY – logD Determination



logD

pH

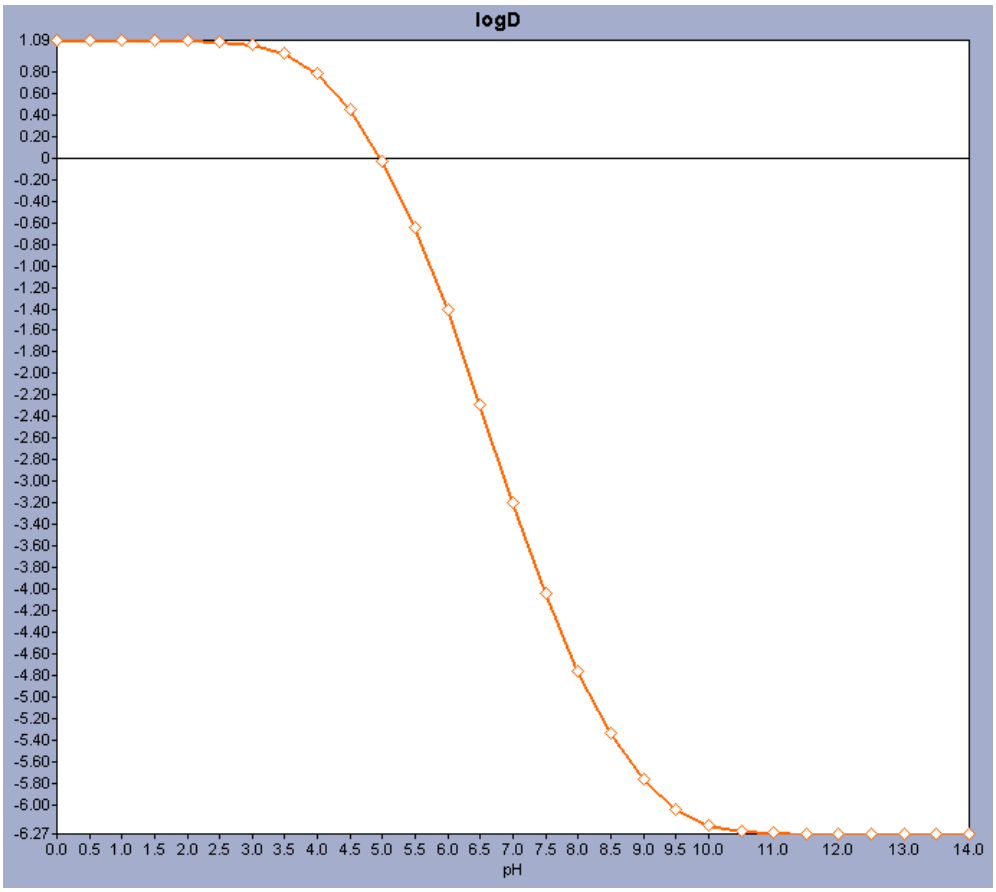
15. MDA pKa Determination – 2 ionizable atoms between pH1-14



Active State

pH

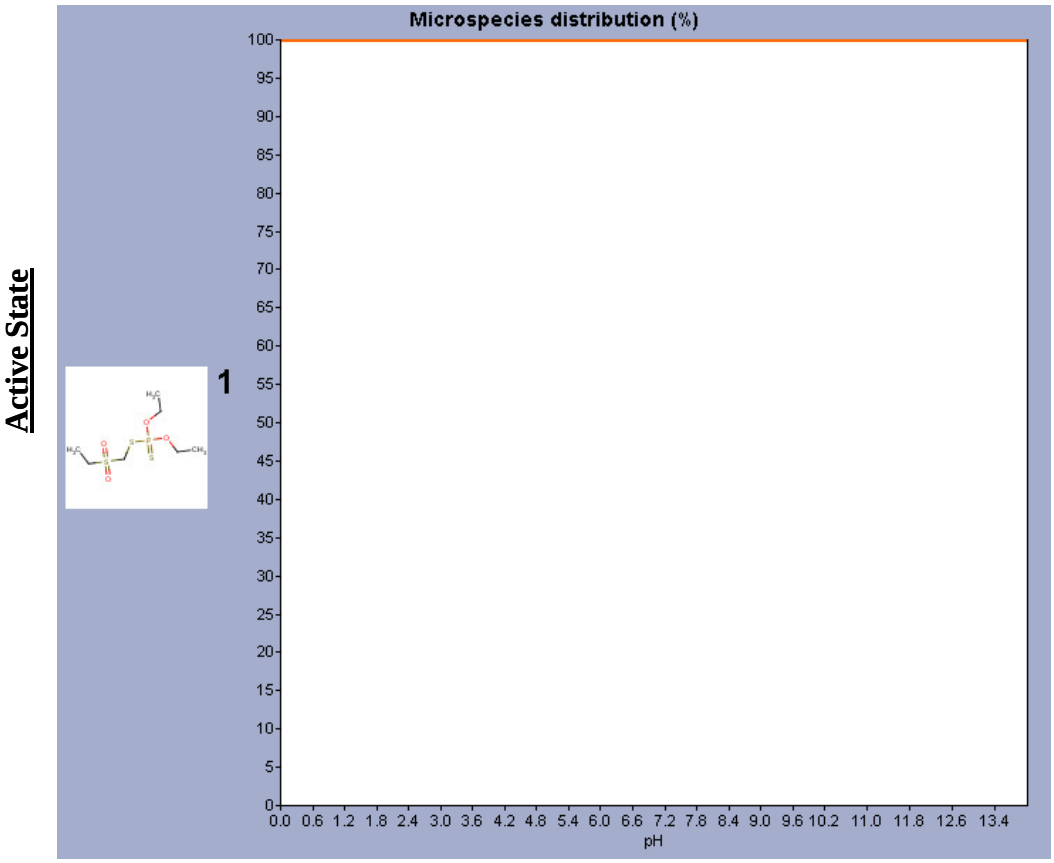
MDA – logD Determination



logD

pH

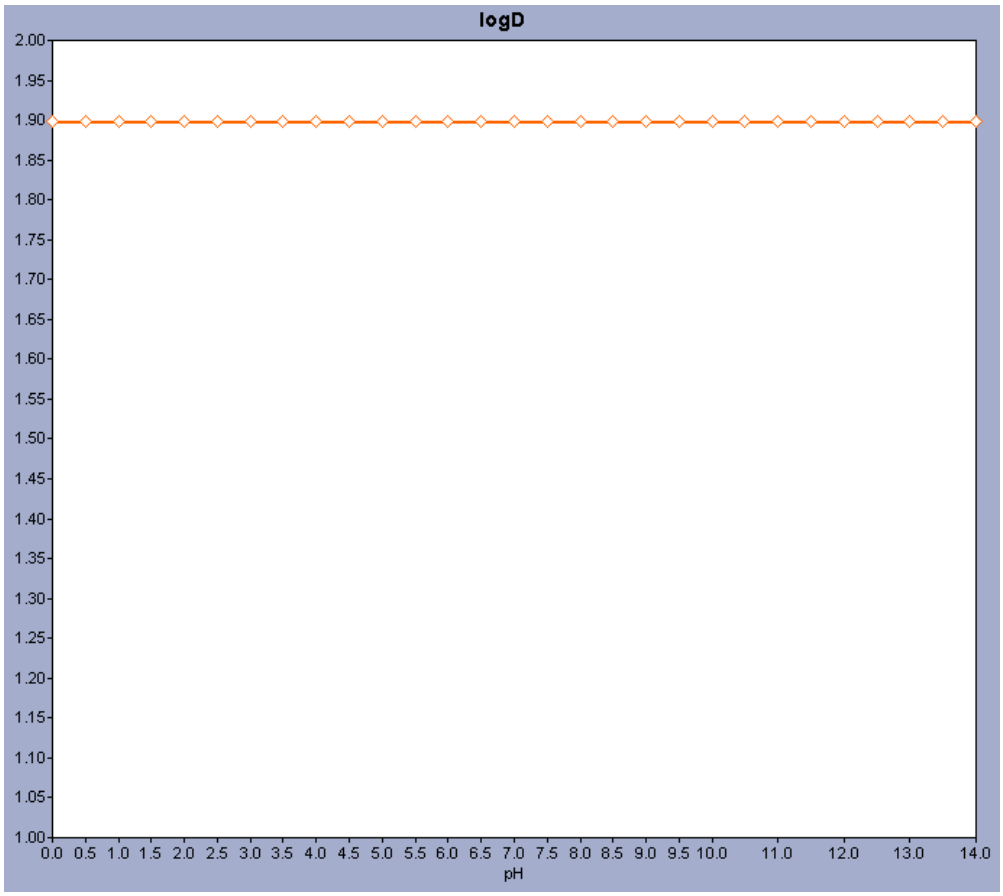
16. Phorate Sulfone pKa Determination – No ionizable atoms between pH1-14



Active State

pH

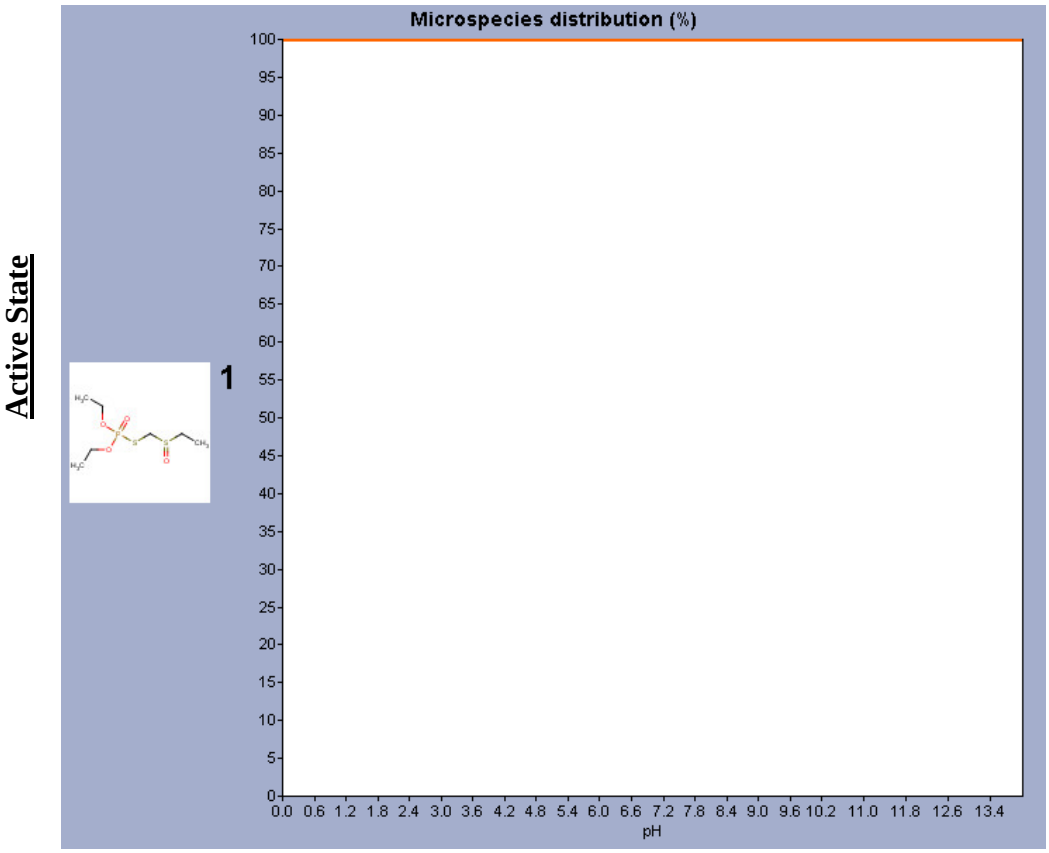
Phorate Sulfone – logD Determination



logD

pH

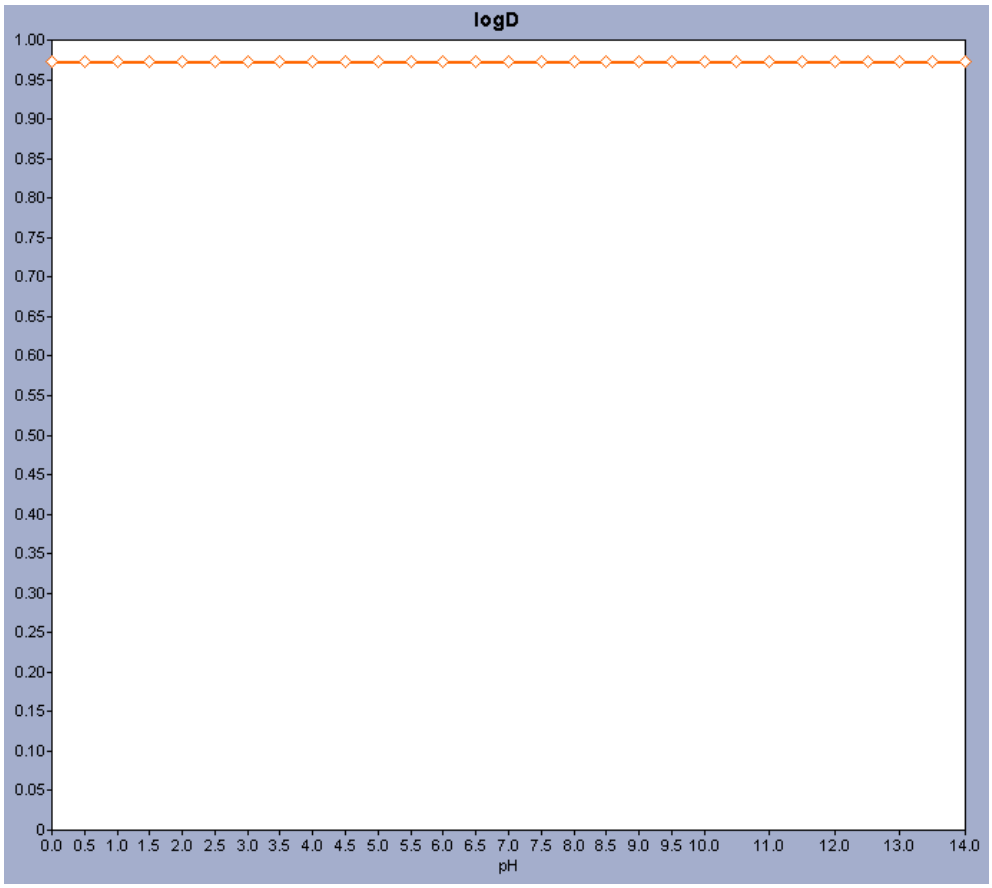
17. Phorate Sulfoxide pKa Determination – No ionizable atoms between pH1-14



Active State

pH

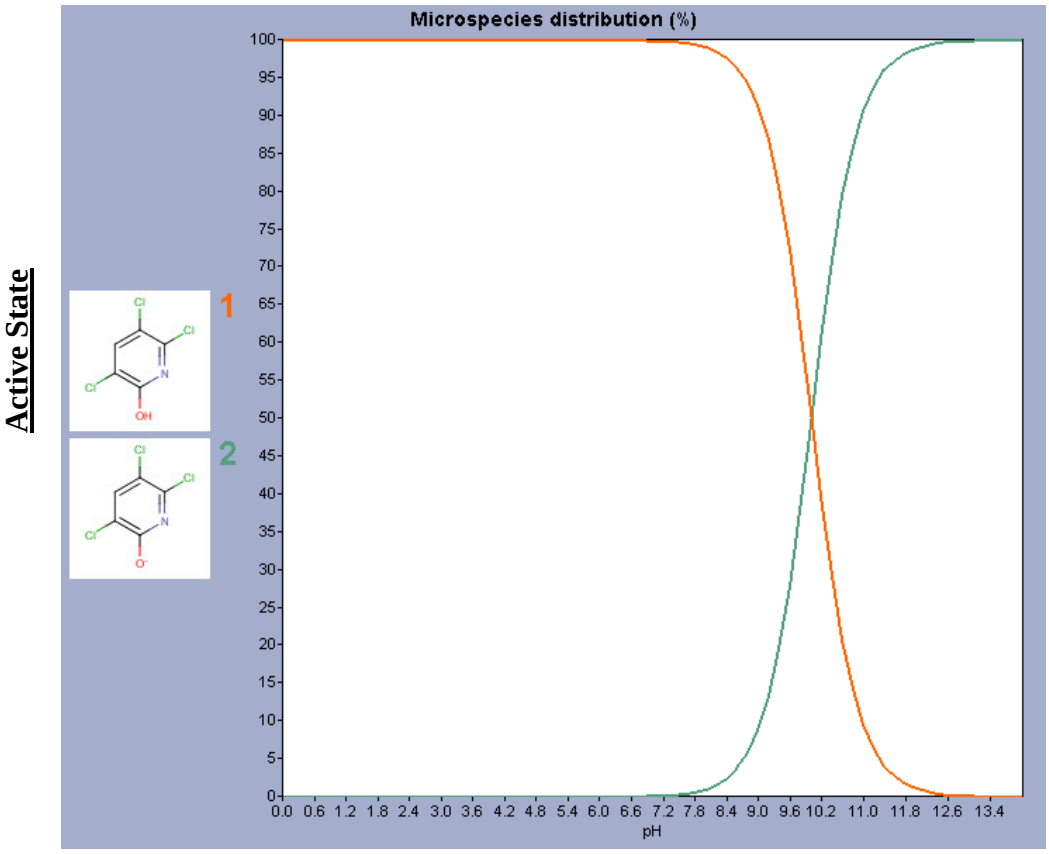
Phorate Sulfoxide– logD Determination



logD

pH

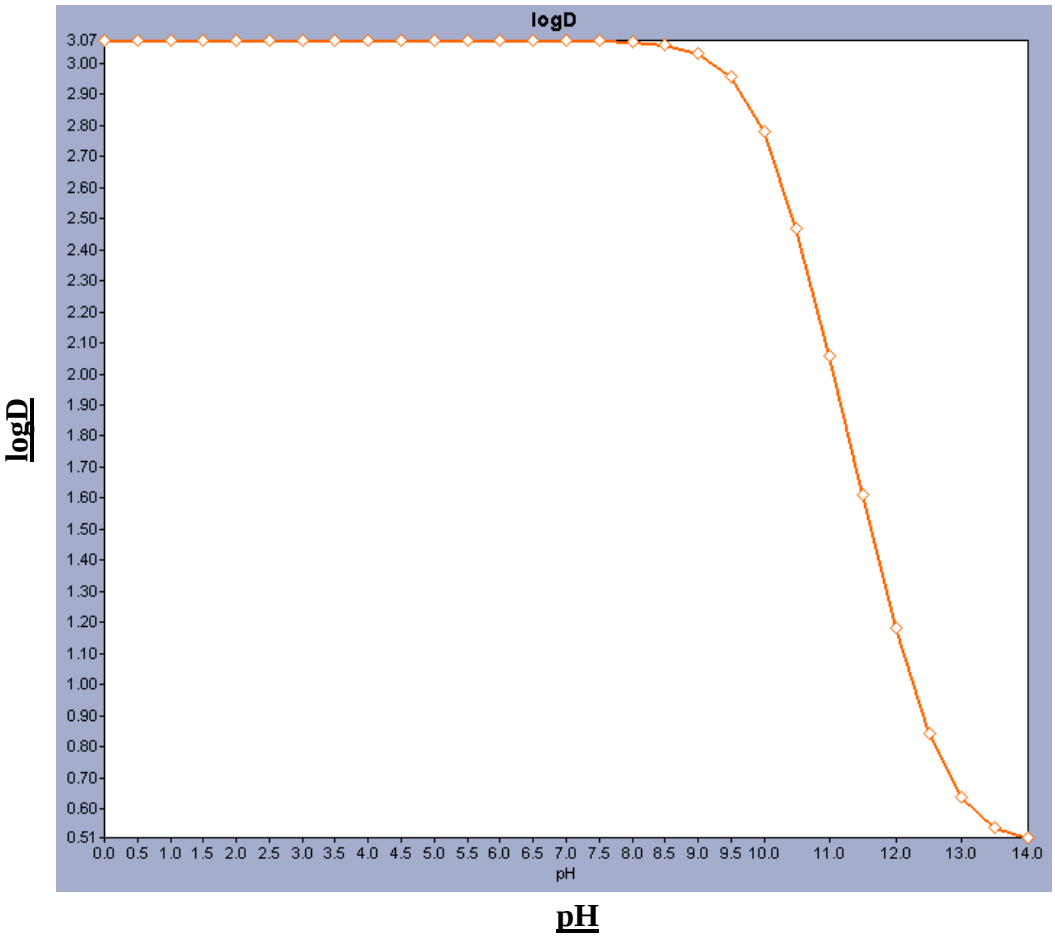
18. TCPY pKa Determination – 1 ionizable atom between pH1-14



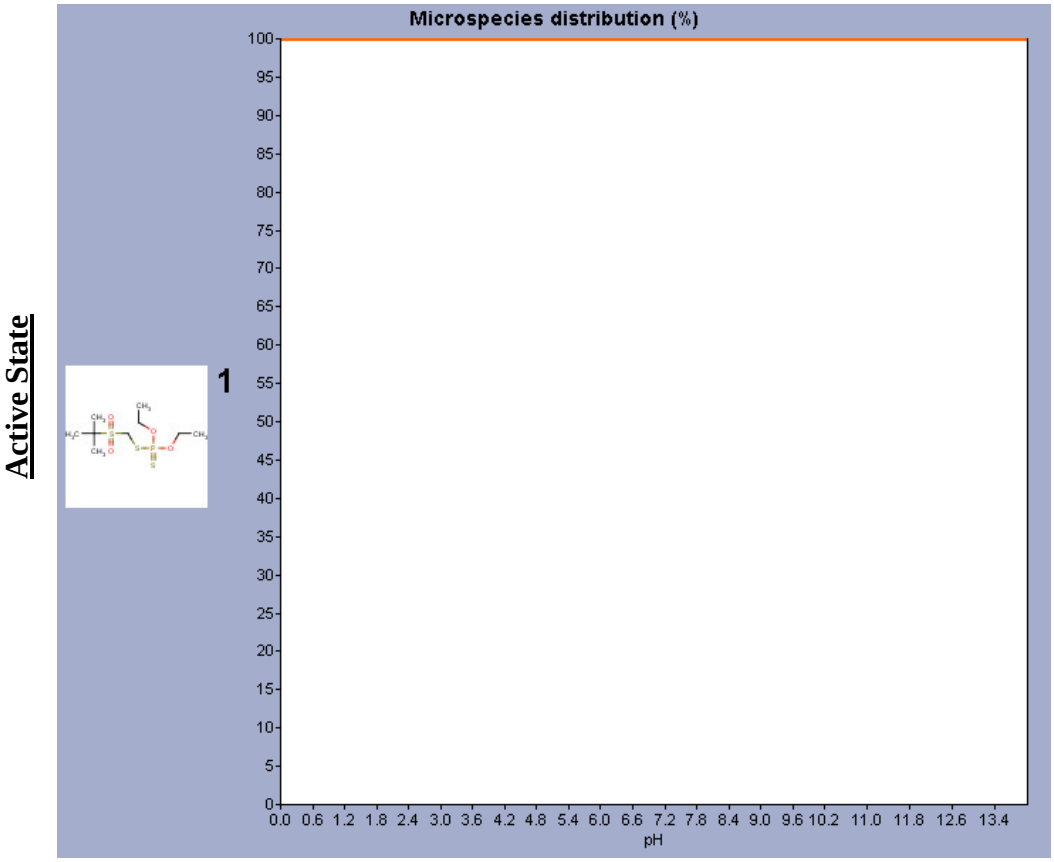
Active State

pH

TCPY– logD Determination



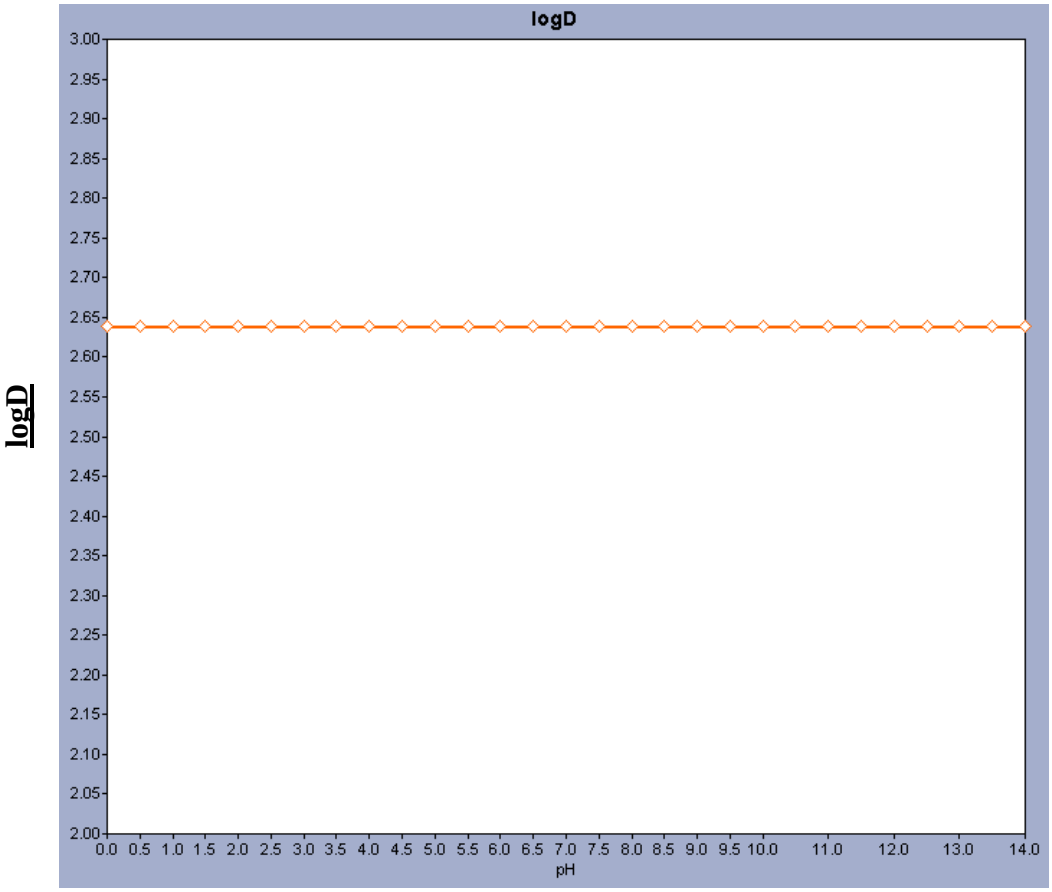
19. Terbufos Sulfone pKa Determination – No ionizable atoms between pH1-14



Active State

pH

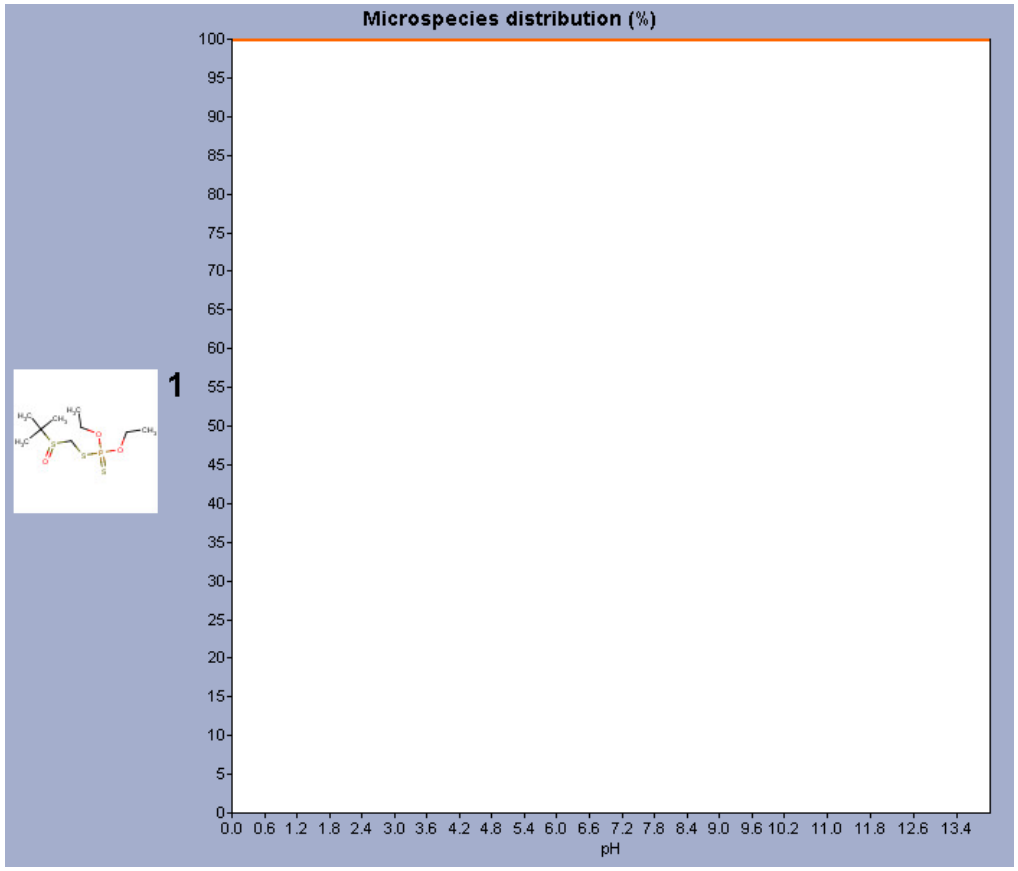
Terbufos Sulfone – logD Determination



logD

pH

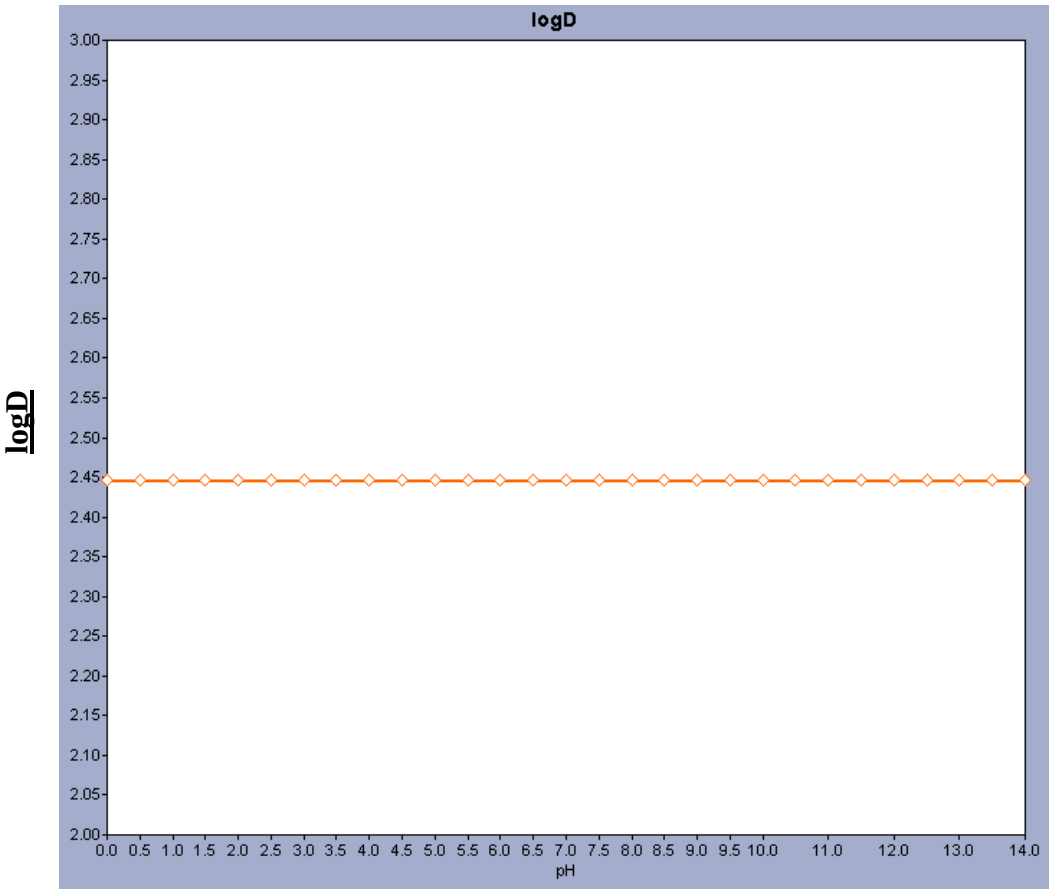
20. Terbufos Sulfoxide pKa Determination – No ionizable atoms



Active State

pH

Terbufos Sulfoxide – logD Determination



logD

pH

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

Conclusions

In this research, multiple extraction methods were evaluated in depth for possible use for high quality environmental trace analysis. Method development can be a long and arduous process; however with specific criteria outlined beforehand, the process can be made logical and systematic. Therefore, specific quality control criteria (accuracy, precision, recovery, etc.) were investigated thoroughly for each analytical method developed. Following quality control criteria will help ensure the generation of reliable data, the ultimate goal for a successful analytical method. Three specific analytical methods were fully developed and validated and are summarized below.

Multiple parent pesticides including organochlorines, organophosphates and pyrethroids were extracted from 1g soil by ASE followed by a secondary liquid-liquid extraction clean-up. From this method, the following conclusions can be made:

- High recoveries of target analytes (60-100%)
- Low detection limits (0.6-13.7ng/g) for target analytes indicate a highly sensitive analytical method that is also congruent with existing published LODs.
- The use of surrogate standards in place in label internal standards can lead to less precise data which was reflected in the poor between-day precision. Additionally, automated standard spiking may also improve precision of the method overall.
- Quantitative assessment using this method is supported by high accuracy observed at the higher end of the calibration curve; however, most of the

target analytes did not fall within +/-15% of the spiked value at the lower end of the calibration curve indicating that at low concentrations, only qualitative assessments should be performed.

- All soil samples should be analyzed for any background concentrations of target analytes that may interfere with development and validation measurements as in the case with chlorpyrifos.

Degradation products of organophosphate (specific and non-specific DAPs) and pyrethroid pesticides were extracted from 1g soil by solid-liquid extraction, specifically shaking and centrifugation, followed by either one of two SPE mechanisms: ion-exchange SPE or molecular imprinted polymer SPE (excluding DAPs). The following conclusions can be made based on these methods:

- Detection limits for the MISPE method (0.66-9.84ng/g) were lower *overall* for the specific OP and pyrethroid degradation products than the ion-exchange method (0.13-28.9ng/g) indicating that MISPE is a more sensitive analytical method for these analytes. The one distinction being IMPY which had lower detection limits with ion-exchange due to low recoveries. DAP LODs with the ion-exchange method were between 0.39-4.22ng/g except for DMP which had a high LOD of 85.67ng/g most likely due to low analyte recoveries (~10%). DAPs were not included in the analysis with the MISPE method due to extremely low recoveries (<5% for 5/6 DAPs).

- Recoveries were similar for both methods with the majority exceeding 70%. DAP recoveries with the ion-exchange method were above 70% except for DMP (~10% recovery).
- The precision measured with QC materials over time was superior with the MISPE method with RSDs lower for between-day, within-day and overall measurements in comparison with the ion-exchange method. Overall, most target analytes were within acceptable limits at both QCL and QCH levels (excluding CFCA, CDCA, IMPY at the QCL level) indicating that they are able to be quantitatively assessed at these levels. DAPs were within acceptable range at both QCL and QCH levels with the ion-exchange method (excluding DMDTP at QCL level) and therefore able to be quantitatively assessed with this method.
- Accurate measurements proved to be problematic for both methods indicating a need for reconsideration of the fortification scheme used for QC materials.
- Matrix effects seen with the ion-exchange method were minimized overall with the MISPE method; the one discrepancy being CFCA which had extreme ion enhancement with MISPE.
- First MIPSE method to include analytes from multiple pesticide classes.

It should be noted that the even with a small recovery of 4% for IMPY with the MISPE method, low detection limits and good precision was obtained. This is exceptional considering the small amount of analyte present. The ability of the MISPE mechanism to achieve higher quality measurements compared with traditional SPE is evident. However, MISPE is definitely limited by its own attributes. The higher selectivity parameter decreases broad-based specificity of the extraction method, a feature that is necessary for multi-class analysis. It is currently trendy to include multiple pesticides from various classes in a single assay (as shown with the parent pesticides). However, perhaps the efficiency-minded direction of analytical method development should be re-considered to instead re-emphasize high analytical quality data.

It is necessary to evaluate all method parameters and determine what is most important to the question needed answered. Is qualitative or quantitative data needed? At what concentrations are analytes expected to be present? How many samples need to be analyzed; *i.e.* how robust is the overall system? How many analytes need to be measured? These are important questions that need to be understood before deciding what analytical method to use for unknown samples.

Understanding this analytical process is necessary for reliable and high-quality data. This will facilitate epidemiological studies investigating whether humans are being exposed to parent pesticides or their degradation products in the environment. These methods will aid in better characterization of exposure, specifically considering multi-chemical

exposures, and subsequent health outcome, the ultimate responsibility of public health practitioners as deemed necessary according to FQPA.

Future Development

Future work should definitely include investigation of soil-pesticide fortification schemes to understand better the discrepancies seen with QCL and QCH materials for both MIPSE and ion-exchange degradation product methods. Perhaps a longer mixing or high-power shaking is needed to homogenize QC pools more effectively.

Additional development of DAPs extraction with MIPSE should be evaluated. DAPs were automatically excluded due to low recoverable quantities and time constraints. Further method parameters should be evaluated to attempt to improve recoveries of DAPs, although this may result in less recovery for the pyrethroid and specific OP degradation products. An individual DAP method separate from the other two groups should be attempted.

DMP proved problematic overall for both methods. An expanded investigation into DMP and its retention behavior would benefit not only this research but additional biomonitoring research that includes DMP (as a metabolite) in OP occupational exposure studies.

Finally, a more comprehensive understanding of matrix effects needs to be evaluated. Although many published analytical methods neglect this topic, it is an important

consideration as it can negatively affect quality of the method. For this research, the presence was discussed but the understanding of why they occur and where exactly they are coming from is inexplicable. Perhaps a better understanding of their presence would lead to reducing their effects and thus improving overall quality.

CHAPTER 5: REFERENCES

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