DEGRADATION OF PENTOBARBITAL IN VARIOUS SOIL TYPES BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY / MASS SPECTROMETRY

By

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ABSTRACT

Pentobarbital is a leading drug for euthanizing large farm animals [Wolfgang et al., 2009]. However, pentobarbital tends to leach into the surrounding soil and become a source of contamination once these euthanized animals are buried. This research was conducted to determine the breakdown rate and extraction efficiency of pentobarbital adsorbed in different types of soil. Additional studies include examining a microbe strain possessing an enzyme capable of breaking down pentobarbital into its metabolites that has leached into the soil.

Solid phase extraction coupled with LC/MS was an efficient method for detecting and quantifying pentobarbital from complex matrices, such as soil. The established method was capable of quantifying 0.5 µg of pentobarbital per gram of soil (500 ppb). The soils were spiked with desired amount of pentobarbital and were analyzed daily and weekly to understand the degradation pattern of pentobarbital. In addition, soil samples were autoclaved at 121°C to determine if any bacteria caused the degradation of pentobarbital in the soil samples. The finding suggests that the degradation of pentobarbital can be due to microbial influences or nature of the soil or possibly both.

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CHAPTER I

INTRODUCTION

Recently the concerns about environmental contaminants such as pharmaceuticals have increased due to the potential risk to health and the environment. Pharmaceutically active compounds are complex molecules with different physiochemical and biological properties and functionalities [Klaus, 2008]. Pharmaceuticals cover a wide range of chemicals, such as over-the-counter and prescription drugs, veterinary drugs, diagnostic agents and vitamins [Nair, 2011]. Pharmaceuticals are contaminating the environment by metabolic excretion, improper disposal, or industrial waste. Contamination by pharmaceuticals can occur as low as parts per in concentration of billion (ppb), or parts per trillion (ppt). However, many studies and analyses have proved that even at this low level, pharmaceuticals have potential adverse human and environmental effects [Halling et al., 1998]. Several pharmaceutical substances seem to persist in the environment, such as estradiol (a steroid and estrogen sex hormone), antibiotics, antidepressants, analgesics and anti-inflammatories [Daughton, 2001]. Barbiturates, a class of depressant appear to belong to this list [Peschka et al., 2006].

1.1 Barbiturates

Barbiturates are a class of pyrimidine-derived drugs that affect and depress the central nervous system. Barbiturates are used as hypnotics, sedatives, anticonvulsants and anesthetics, although they are most familiar as 'sleeping pills'. The properties of barbiturates

depend upon the side groups or chains attached to the ring. Pyrimdine forms the basic structure of the barbiturates. Pyrimidines are nitrogen containing heterocyclic aromatic compounds. They are planar and include several nucleic acid constituents such as cytosine, thymine and uracil. Figure 1 shows the nitrogenous bases commonly found in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Figure 2 shows pyrimidine forms the basic structure of barbiturate.

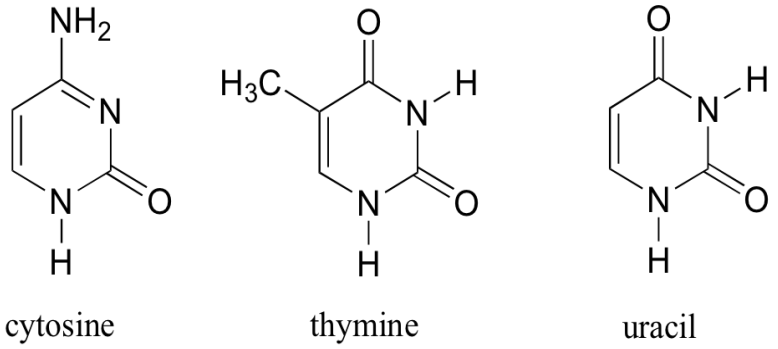


Figure 1: Nitrogenous bases that are pyrimidine derivatives found in DNA and RNA**.**

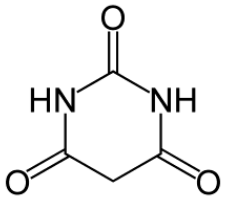


Figure 2: Basic structure of barbiturate

1.1. a. History of Barbiturates

Nobel Prize winner Adolf von Baeyer synthesized the first barbiturates in 1864. In 1879, french chemist Edouard Grimaux perfected the synthesis process [Lopez- Munoz et al., 2005]. Diethyl-barbituric acid is the first cinical form of barbituates synthesized by Conard and Guthzeit. German companies E. Merck and F. Bayer introduced barbiturates commercially as a hypnotic drug called "barbital" [Lopez-Munoz et al., 2005]. Barbiturates are derivatives of barbituric acid. They work by depressing the central nervous system in a dose dependent fashion. Barbituric acids are synthesized from malonic acid and urea. While barbituric acid has no pharmacological activity, barbital derived from it has a sedative hypnotic property [Dasgupta, 2014].

1.1. b. Synthesis of Barbituric Acid

Barbituric acids are synthesized from malonic acid and urea by a condensation reaction resulting in the release of H_2O (dehydration) and the heterocyclic barbituric acid (Figure 3). Substituting required side chains on the ring produces the pharmacologically active barbiturates.

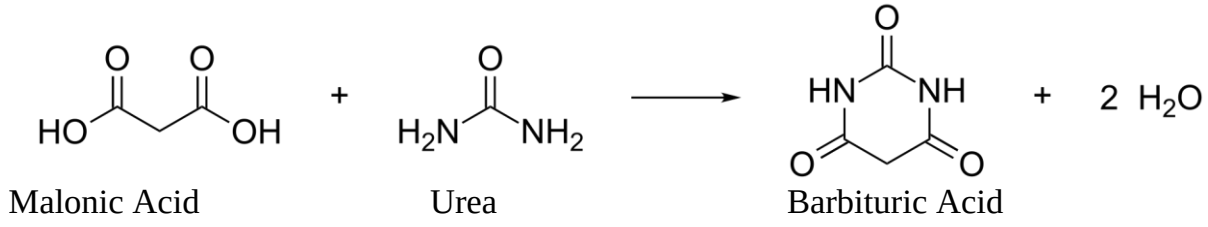


Figure 3: Synthesis of barbituric acid

The properties of the various barbiturates depend upon the side groups attached to the ring [Oak Pharmaceuticals, 2012]. There are over 2,500 derivatives of barbituric acid have been synthesized and approximately 50 of them have been marketed. Figure 4 shows some of the commercially available barbiturates. Approximately 12 different barbiturates are used medically worldwide [Dasgupta, 2014].

1.1. d. Mechanism of Action

Neurons are the specialized cells compose the human nervous system. Neurotransmitters are the chemicals located in the brain, which allow the transmission of signals from one neuron to the next across synapses. This is essential for the normal function of both the central and peripheral nervous systems [Boeree, 2003]. There are excitatory or inhibitory neurotransmitters. γ -aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS) . Neurotransmitters are released from an axonal end of one nerve cell where they diffuse across a gap to the dendrite end of another nerve cell. This is identified as the synaptic cleft. To carry out an action specific receptors molecules on the surface of the receiving cell attaches the neurotransmitter and consequently send a signal inside the cell [Voet et al., 2008].

Barbiturates affect the major inhibitory neurotransmitter GABA [Boehm et al., 2004]. In the mammalian nervous system, GABA is the major inhibitory neurotransmitter often referred to as “nature’s valium-like substance” [Neurogistics, 2015]. The GABA receptor has a ligand-gated receptors structure. While the mechanism of action is ongoing, barbiturates appear to act by increasing the duration of the channel opening of the GABA receptor [Olsen et al., 1999].

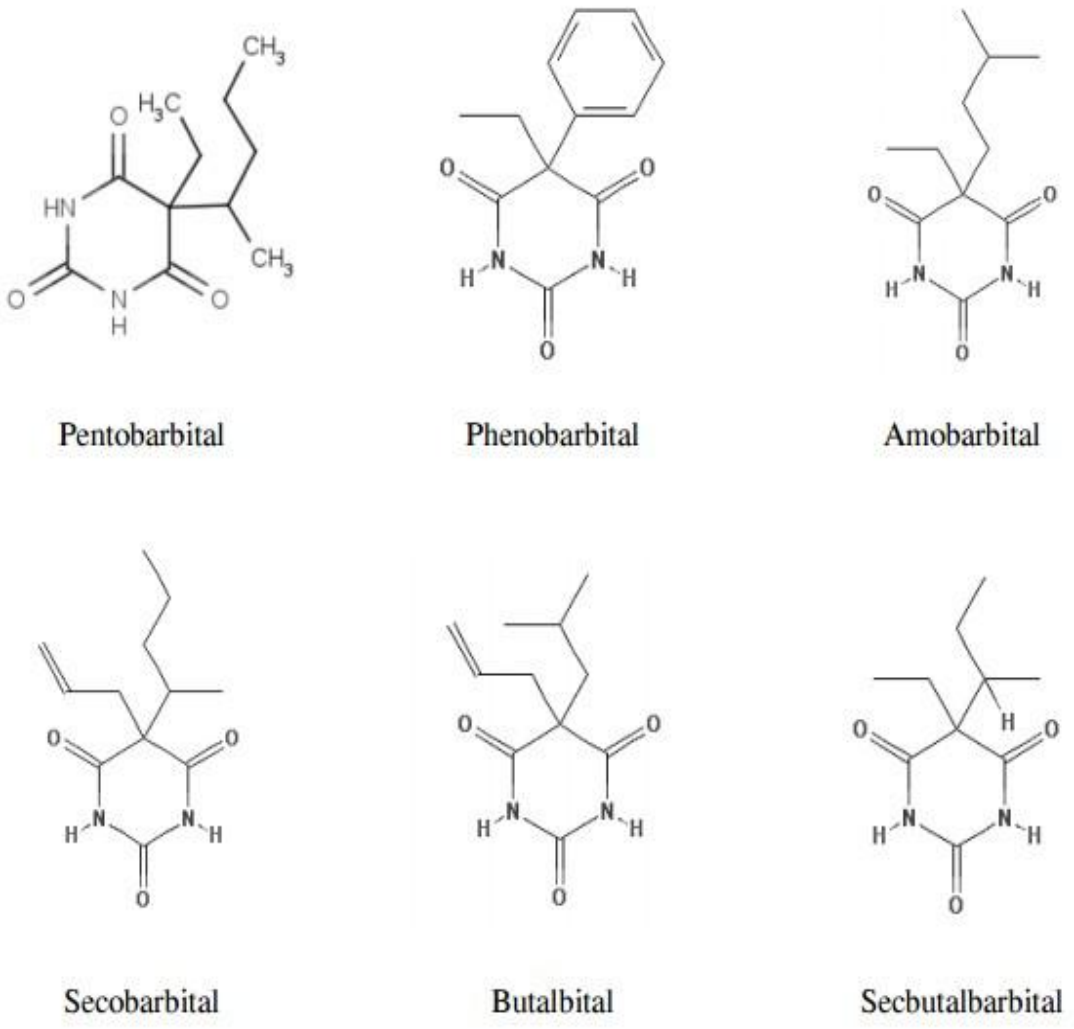


Figure 4: Derivatives of barbiturates that are commonly used taken from Public Chemical Database [NCBI, 2013].

Barbiturates bind to the β-subunit of the GABA-ion receptor complex and cause a change in conformation of the ion channel. The conformational change allows more chlorine ions into the intracellular matrix of the cell.

Barbiturates have the ability to pass through the blood brain barrier of the human body. The chloride ion influx into the cell is enhanced if barbiturate derivatives that are lipophilic enough to pass the blood brain barrier. In addition, lipophilic barbiturates will inhibit the firing of the action potential to the next cell [Olsen et al., 1999].

1.1. e. Categories of Barbiturates

Several thousand derivatives of barbiturates have been synthesized with widely varying effects and flexible durations of action. Barbiturates are divided into categories based on time needed to produce an effect and the period those effects last. The onset of action varies depending to the lipid solubility of the barbiturate. A highly lipid-soluble the barbiturate will distribute faster through the tissues, especially the brain, liver and kidneys [Oak Pharmaceuticals, Inc, 2012; American Society of Health Systems Pharmacists, 2009].

Barbiturates can be classified as ultra short, short, intermediate, and long acting.

- "Ultra short-acting" barbiturates produce anesthesia within one minute after intravenous use. Thiopental is an ultra short acting barbiturates.
- "Short-acting" and "intermediate-acting" barbiturates take effect within 15 to 40 minutes and last up to six hours. They are used for sedation or to induce sleep. Intermediate acting barbiturates are typically used as hypnotics. Pentobarbital and secobarbital are an example of short acting barbiturates.

Amobarbital and butabarbital are classified as intermediate acting barbiturates.

- "Long-acting" barbiturates take effect in an hour and last up to 12 hours. They are used primarily for sedation and the treatment of seizure disorders or mild anxiety. Phenobarbital is classified as a long- acting barbiturate.

1.2 Pentobarbital Properties

Pentobarbital is classified as a fast-intermediate sedative-hypnotic drug. It is highly lipophilic and penetrates the blood brain barrier quickly, limited only by the rate of cerebral blood flow [American Society of Health Systems Pharmacists, 2009]. Maximum CNS suppression occurs if pentobarbital is administered orally within 15 - 60 minutes and within a minute if administered intravenously [American Society of Health Systems Pharmacists, 2009]. If administered orally, the half-life of the distribution phase is approximately one to four hours and only 15 minutes if administered intravenously. The elimination known as the "beta phase" occurs in approximately 35 - 50 hours [American Society of Health Systems Pharmacists, 2009]. OAK pharmaceuticals marketed as sodium pentobarbital as Nembutal ® Sodium Solution. Adults can take up to five days to eliminate pentobarbital [Kwan and Brodie, 2004].

1.2. a. Synthesis of Pentobarbital

Pentobarbital synthesis is a condensation reaction between a substituted malonic ester (1-methyl butyl-ethyl malonic ester) and urea followed by hydrolysis to give the resulting barbital compound [Neumann, 2004]. Other names of pentobarbital include 5-ethyl-5-(1-methylbutyl)-barbituric acid and 5-ethyl-5-(1-methylbutyl)-2, 4, 6,-trioxohexahydropyrimidine. Figure 5 illustrates the synthesis of nembutal, a brand name for pentobarbital. Table 1 briefly describes the structure and the properties of pentobarbital.

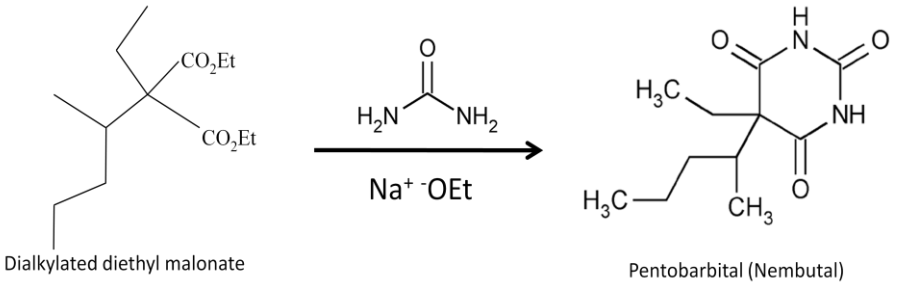
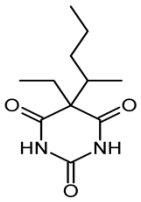


Figure 5: Synthesis of pentobarbital.

Table 1: Structure and properties of pentobarbital

	
IUPAC Name	**5-ethyl-5-pentan-2-yl-1,3-diazinane-2,4,6-trione**
Other Names	**5-ethyl-5-(1-methylbutyl)-barbituric acid**
	5-ethyl-5-(1-methylbutyl)-2,4,6,-trioxohexahydropyrimidine
Brand Name	**Nembutal**
Common Form	**Sodium Pentobarbital**
Molecular Formula	**$C_{11}H_{18}N_2O_3$**
Molecular Weight	**226.27 g/mol**
pK_a	**7.8**
Solubility in Water	**679 mg/L in 25°C**
Melting Point	**129.5°C**
Classification	**Fast Intermediate Barbiturates**
Legal Status	**Class II**
Onset of Action	
Few seconds intravenously	**15 - 60 minutes orally**
Duration of Action	
15 minutes if administered intravenously	**1 - 4 hours if administered orally**
Dosage	
Maximum Daily Dose:200 mg	**Hypnotic Dose: 100 mg**	**Lethal Dose (Human): 9 - 10 g**

1.2. b. Pentobarbital as a Euthanasia Drug

Although the overall usages of barbiturates derivates have declined, pentobarbital is still used extensively throughout the United States for medicinal purposes. Pentobarbital is used often in the medical field as a preoperative depressant and as an emergency treatment for seizures. However, its popularity has shifted from the medical field to the veterinary field. Pentobarbital is the leading method for euthanizing large farm animals [Wolfgang et al., 2009]. Pentobarbital causes death by paralyzing the brain stem and medulla [Kiran et al., 2002]. Euthanizing sick or injured animals with lethal pentobarbital injection is a more humane method of killing than other types of euthanasia such as shooting. In veterinary medicine, lethal doses range from approximately 30 - 40 grams of pentobarbital to euthanize a mature cow or horse [Wolfgang et al., 2009]. While administration of pentobarbital is a humane method of euthanizing large farm animals with no detrimental effect, a problem arises in the disposal of the carcass.

Several options are used to dispose of euthanized animals such as horses and cows, including burial, composting, rendering, cremations and landfills [Cottle, 2009]. Some of these methods are not cost effective and illegal too [The Humane Society of the United States, 2013]. However, it is essential to dispose of euthanized carcasses properly. In 2003, the FDA issued a warning stating "euthanized animals must be properly disposed by deep burial, incineration, or other method in compliance with the state and local laws to prevent consumption of carcass material by scavenging wildlife" [Bonhotal et al., 2012].

1.2. c. Ground Water Contamination by Pentobarbital

Water-soluble contaminants are transported by vertical and horizontal groundwater flow [Post et al., 2007]. Recent studies have shown the problematic environmental effect of burying carcasses euthanized with pentobarbital. The research is inadequate, but preliminary results suggest buried carcasses leach pentobarbital from the animal tissue into the surrounding soil and water supply [Eckel et al., 1999]. Soil contamination is often related with the contamination of groundwater. In soil, water moves vertically, at a rate largely determined by soil texture, where excess rain is absorbed into the deeper layers, thereby generating groundwater [Vicent et al., 2011]. By contrast, lakes and river systems drive horizontal groundwater flow. Groundwater pollution is very difficult and expensive to manage. As a result leaching of contaminants into groundwater should be prevented [Valentin et al., 2013]. In Jacksonville, Florida, ground water was collected from a water supply near a landfill, which received wastes around late 1960s. The ground water tested positive for pentobarbital residues more than 15 years following the end of the usage of the landfill. The water supply was tested again after a period of 22 years following the time it received wastes. Pentobarbital was present in the water supply and the concentration was 1 µg/L, which corresponds to 1 ppb [Eckel et al., 1999].

1.3 Cases of Secondary Toxicosis

Veterinary case reports the deaths wild animals, pets and scavenging birds involving secondary contamination. [Kaiser et al., 2010; National Library of Medicine, 2010; Bonhotal, et al., 2012; Cottle et al., 2009]. In 2003, the FDA issued a warning stating "euthanized animals must be properly disposed by deep burial, incineration, or other method

in compliance with the state and local laws to prevent consumption of carcass material by scavenging wildlife" [Bonhotal et al., 2012]. In addition to poison by scavenging, a few reports confirm that animals can also be poisoned by meat fed to them supplied from euthanized animals. In one case, three tigers in Heidelberg Zoo in Germany were poisoned after ingesting contaminated meat. The contaminated meat was a horse euthanized with pentobarbital [Jurczynski and Zittlau, 2007]. In another case, an FDA research team conducted a study collaborating with the Center for Veterinary Medicine on the safety of feed products for animals. The feed products supported the presence of pentobarbital in dog food samples. The presence of pentobarbital in the feed products were confirmed using gas chromatography / mass spectrometry (GC/MS) and liquid chromatography / mass spectrometry (LC/MS) [Adam and Reeves, 1998; Heller, 2000].

One study examined the pentobarbital residues in compost piles containing euthanized carcasses **[**Kaiser et al., 2010**]**. The compost samples tested positive for pentobarbital residues within days of burying the euthanized carcass. After a while, additional samples from the compost pile confirmed the increase in concentration of pentobarbital [Cottle et al., 2009]. Another concern is the time it needs for pentobarbital to break down, if at all. In a case of secondary poisoning from pentobarbital, two dogs found an unburied horse carcass in a ravine. The horse had been euthanized with pentobarbital and was not buried properly. The carcass was dumped in the ravine more than two years earlier [Kaiser et al., 2010]. One of the dogs ingested a lethal dose, which is reportedly 85 mg/kg for dogs. Another concern is scavenging birds. In recent years, more 140 bald and golden eagles have been deceased after ingesting pentobarbital- tainted carcasses [Krueger, 2002].

1.4 Types and Properties of Soil

Soils are the thin, outermost layer of the Earth's crust and an essential element in the ecosystem. Soils are composed of layers or horizons and are a complex mix of minerals, air, water, and countless microorganisms [USDA, 2013]. Soil originally formed from parent material: a deposit at the Earth's surface, which is gradually changed by sun, water, wind, and microorganisms into soil [Soil Science Society of America, 2016]. Soils are scientifically described based on: color, compaction, moisture content, organic content, pH, structure and profile. Dark color soils are considered fertile with high organic matter and elevated levels of nitrogen content. A loosely compacted soil helps to absorb and retain water, releasing it slowly, making the soil productive [Soil Science Society of America, 2016]. The organic content of soil greatly influences the soil properties including the plant, animal and microorganism populations present. Decomposing organic material provides many necessary nutrients to soil inhabitants [Bot, 2005]. Soil pH is typically around 6.0 to 7.4. Soil profile provides the horizons or layers of soils, which are top soil, subsoil and parent material [USDA, 2013]. Soil particles shape and arrangement determines the soils porosity. Porosity is the measure of empty space or void space between soil particles. These void spaces are used for groundwater movement and nutrient storage. Both sand and clay have high porosity [Soil, 2015]. However, not all the water stored in pore spaces becomes part of groundwater. Water adheres to soil particles and surface tension, cohesion, or adhesion helps to forms a thin coat around a soil particle. Specific yield or drainable porosity measures the amount of water that drains and becomes part of groundwater [Soil, 2015]. Permeability is a measure of the ability of a soil or rock to transmit water. A material is more permeable if the pore space

is large. The soil acts as a natural filter and has ability to reduce the severity of groundwater contamination known as soil attenuation. [Soil, 2015].

Soil samples utilized in this study are potting soil, topsoil and sand. Topsoil is the upper layer of the soil containing the most organic matter and microorganisms. Topsoil has a pH around 6.0 - 6.7. Topsoil layers commonly range from two to ten inches thick [Koenig, 2010]. Sand is a naturally occurring granular material that contains mineral particles. Sand is the largest soil particle mixed in different proportions to compost earth. Soils with large amounts of sand have big spaces between the particles. They do not hold water or nutrients well. Sand does not react with other chemicals and sandy soils do not stick together very well. Plant roots cannot hold onto this soil. However, the big spaces do allow air into the soil [USDA, 2015]. Potting soil or potting mix is a growth medium for plants, herbs and vegetables. Potting soil holds moisture, nutrients and air around the plant roots, acting as a reservoir for these critical elements [Reid, 2015]. The key ingredients for potting soil are sphagnum peat moss, vermiculite or perlite, and aged compost products [Reid, 2015]. Some potting soils contain limestone to balance the soil pHs. Sphagnum peat moss holds moisture in the soil. Perlite separates the fibers in the peat moss so the soil is more porous. Vermiculite has the same function but holds more water than perlite [Miracle-Gro, 2013].

1.5 Persistence of Pentobarbital in Soil

Pharmaceuticals are entering the environment from diverse sources at an alarming rate. There is a concern over the consequence pentobarbital euthanized carcasses have on the environment since increasing number of animals are euthanized each year. According to Cornell Waste Management Resources, around 900,000 horses must be disposed of annually

in the U.S. [Bonhotal et al., 2012]. However, FDA regulations limiting the sources of rendering plants have created problems in the disposal of pentobarbital-containing carcasses[Federal Meat Inspection Act, 2009]. Disposal by burial or composting is a cost effective and growing method. However, recent studies have illustrated the environmental effect of burying carcasses euthanized with pentobarbital. Research suggests burial can cause leaching of pentobarbital from euthanized animal tissue into surrounding soil [Wolfgang et al., 2009].

The persistence of a pharmaceutical in soil or sediment depends primarily on its photo stability or photolysis, binding, and sorption capabilities along with its decay rate [Diaz-Cruz et al., 2003]. Photolysis is a chemical reaction in which chemical compounds are broken down by photons. For example, photolysis of reactive bromine species such as Br_2 and BrCl leads to the formation of bromine radicals (Br*) and the subsequent destruction of ground level ozone (O_3) in Polar Regions [Foster et al., 2001]. Pentobarbital is not subject to photolysis due to its lack of chromophores or its inability to absorb light in the visible spectrum [Lyman, 1990]. Pentobarbital has a high degree of mobility, smaller absorption in the soil and a higher degree of solubility in water.

Pentobarbital's high degree of mobility in the soil is based on the soil's organic carbon-water partitioning coefficient or K_{oc}. Pentobarbital has an estimated value of 28 K_{oc} [Hansch et al., 1995]. K_{oc} is a measure of the tendency of a chemical to bind to soils, corrected for soil organic carbon content. K_{oc} values can vary substantially, depending on soil type, soil pH, the acid-base properties of the substance and the type of organic matter in the soil [Weber et al., 2004]. High K_{oc} values indicate soil absorbing a high degree of

contaminant and the contaminant are considered less soluble in water and therefore less mobile. A lower K_{oc} value correlates to a smaller amount of contaminate absorbed in the soil and a higher degree of the contaminants solubility in water and thus more mobility [Kerle et al., 2007]. For example, reported K_{oc} values for the herbicides clomazone and sulfentrazone are 300 and 26. The herbicide sulfentrazone has a high degree of mobility and smaller absorption to soil than the herbicide clomazone [Cerdeira et al., 2015].

Compounds that sorb weakly to soil are not highly available for microbial degradation and plant uptake. The reported pK_a of pentobarbital is 7.8, which suggests that it exists as an anion form in the environment. As a result, pentobarbital is less likely to be absorbed by soil making it more susceptible to uptake by ground water [Kerle et al., 2007; Wollweber, 2008; Doucette, 2000]. Higher mobility in soil and solubility in water facilitates the leaching of pentobarbital into ground water.

According to Hazardous Substances Data Bank, biodegradation data for pentobarbital are not widely offered [HSDB]. In general, research is very limited on the microbial degradation of barbiturates. Recent work by Dr. Mary Farone at Middle Tennessee State University confirms that certain soil microorganisms are capable of degrading barbiturates **[**Aerobic Decomposition, 2011]. Two different bacteria isolated directly from soil showed enhanced growth in barbital-containing media, indicating pentobarbital degradation. Aerobic soil biodegradation is a major pathway of degrading pharmaceutical drugs in the soil. Pharmaceuticals degrade more rapidly in soil because of the diverse microorganisms [Nezha et al., 2013].

In 1951 and 1952, Wang and Lampen from Case Western Reserve University, and Hayaishi and Kornberg of the National Institutes of Health respectively [Soong et al., 2002] discovered a soil bacterium, Rhodococcus erythropolis JCM 3132, capable of successfully metabolized pyrimidines. Hayaishi and Kornberg stated that bacterial enzymes were involved in metabolism of pyrimidine [Hayaishi and Kornberg, 1952]. The soil bacterium, *Rhodococcus erythropolis* JCM 3132 possessed an enzyme "barbiturase" which was a key enzyme to metabolize barbituric acid to urea and malonic acid [Hayaishi and Kornberg, 1952]. The precise way in which barbituric acid was metabolized was undetermined [Hayaishi and Kornberg, 1952].

1.6 Pyrimidine Metabolism

Pyrimidine metabolism can occur via reductive or oxidative pathways (Figure 6). In humans and mammals, pyrimidine and its derivatives such as pentobarbital are metabolized in the liver by reductive pathway (Figure 6A) and excreted in the urine as the inactive metabolite hydroxypentobarbital. Hydroxypentobarbital results from the oxidation of the 1-methylbutyl substituent [American Society of Health Systems Pharmacists, 2009]. The reductive pathway for pyrimidine degradation generates NH_3 and CO_2. In addition, the pathway generates β-alanine from uracil, and β-aminoisobutyric acid from thymine. According to the AHFS Drug Information Database, 40 - 50% of a normal hypnotic dose (100 mg) is excreted in the urine as the inactive metabolite hydroxypentobarbital [American Society of Health Systems Pharmacists, 2009].

In microorganisms pyrimidines such as pentobarbital are metabolized via an oxidative pathway (Figure 6B). Studies done by Soong et al. in 2001, show that in the

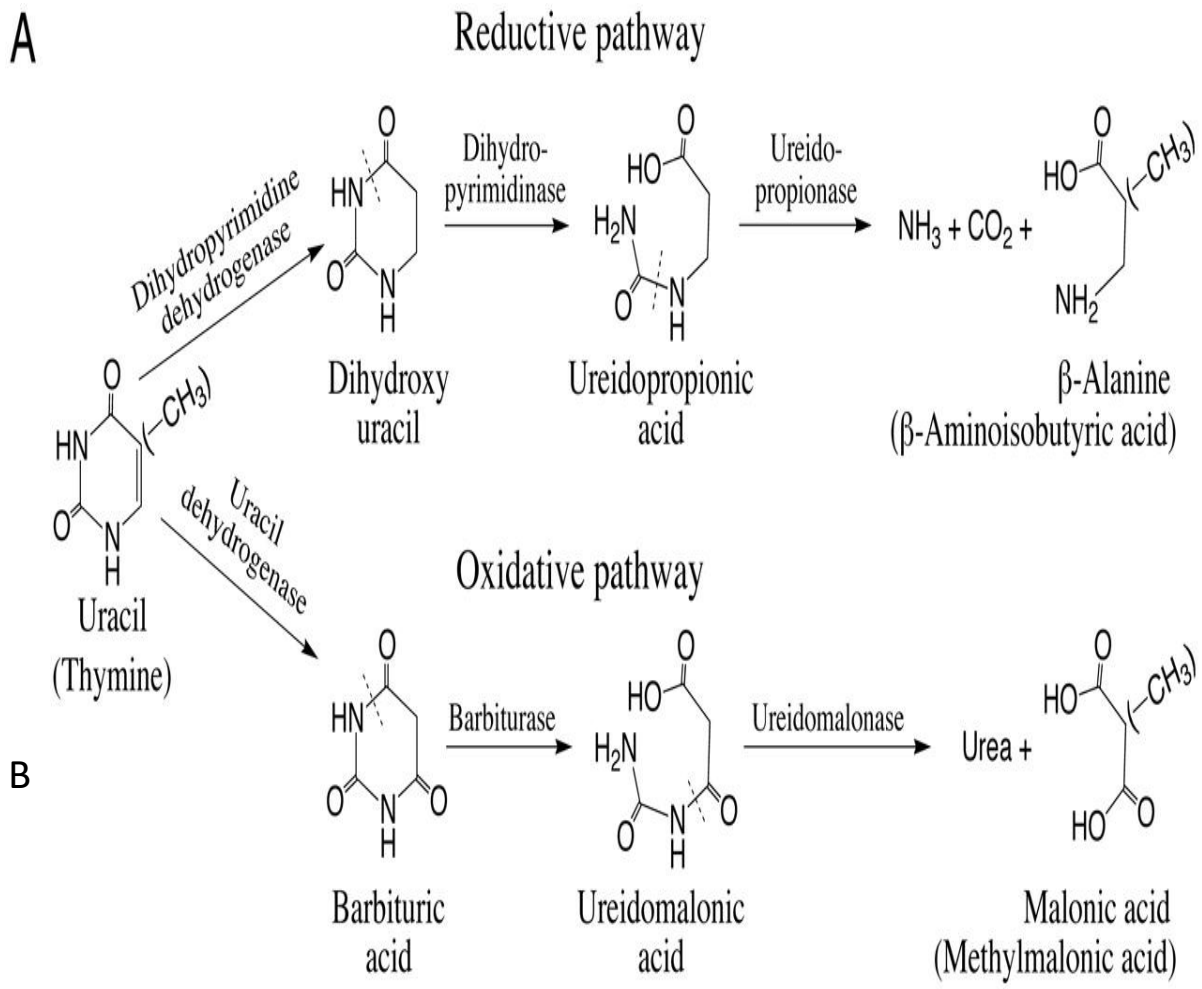


Figure 6: A: Reductive pathway of pyrimidine 2001. B**:** Oxidative pathways in microbial metabolism of pyrimidine [Chee-Leong Soong et al. in 2001].

oxidative pathway barbituric acids are converted to ureidomalonic acid by using the enzyme barbiturase. Finally, ureidomalonic acid is converted to malonate and urea using the enzyme ureidomalonase [Soong et al., 2002]. In their work, they were able to purify and characterize the enzyme barbiturase.

1.7 Methods of Detecting Pentobarbital

Chromatography is a method used for separating organic and inorganic components of a mixture. This separation occurs based on the interactions of the sample with the mobile and stationary phases [Betancourt, 2015]. Compounds in the gaseous or liquid states are primarily identified using chromatographic techniques. The equilibrium between the stationary phase and the mobile phase is the fundamental principle of chromatography [Ju-Seop Kang, 2012]. The stationary phase usually composed of an inert material coated on the inside of a hollow stainless steel tube known as a column.

The mobile phase is a liquid or gas. The sample or mixture is dissolved in the mobile phase and then carried through the column under pressure. Usually LC/MS utilizes a reverse phase chromatography technique where metabolites bind to the column by hydrophobic interactions in the presence of hydrophilic solvent [Watson & Sparkman, 2007]. Elution occurs via a hydrophobic solvent. Common mobile phases include miscible combinations of water with various organic solvents acetonitrile or methanol [Ju-Seop Kang, 2012].

The most common organic solvents are acetonitrile and methanol. Retention time usually verifies the identity of the chemical of interst. A definitive identification is possible using analysis by a mass spectrometer. A mass spectrometer is an instrument often

connected to a liquid or gas chromatography in tandem to characterize sample based on the mass to charge ratio of the species [Rouessac and Rouessac, 2007]. The FDA has been using LC/MS for detection of contaminants in animal food products [Pet Food Regulation, 2016]. LC/MS offers a quicker and more flexible analysis since samples do not require derivatization as is often the case in GC/MS [Heller, 2000]. Pentobarbital has been detected in the breast milk of lactating mothers. The analysis of the presence of pentobarbital in breast milk revealed that after 32 days of normal use of the drug by the mother, pentobarbital was detectable in the breast milk. Pentobarbital concentration was found to be 0.17 μg/mL after a dosage 19 hours earlier. The detection technique used in the study was LC/MS [Briggs et al., 1994]. Research conducted by Baker et al. detected different pharmaceutical products such as benzodiazepines, morphine and barbiturates in waste water and sludge samples using LC/MS [Baker, 2011]. Research conducted by Hori et al., in Japan investigated a method for screening, identifying, and quantifying salicylic acid, acetaminophen and barbiturates. They detected pentobarbital in serum employing liquid chromatography/electrospray mass spectrometry (LC/MS) coupled with solid phase extraction [Hori et al., 2005].

Since small amounts of analytes are often involved in liquid chromatography / mass spectrometry (LC/MS), a concentration step is usually carried out before analysis. The concentration step uses solid phase extraction (SPE). Solid phase extraction (SPE) is a sample preparation technique used for complex matrices such soil, sludge and wastewater. Solid phase extraction is an excellent technique for sample extraction, concentration, and cleanup [Russo et al., 2015]. Research work conducted to analyze barbiturates in wastewater

and sewage samples in Spain utilized solid phase extraction coupled with liquid chromatography / mass spectrometry [López et al., 2010]. Although there are some studies detailing the fate of barbiturates, there remains a need for detecting barbiturates in environmental samples. Additional miscellaneous methods utilized in the study are ELISA, an abbreviation for "enzyme-linked immunosorbent assay." [Bio-Rad, 2016] ELISA analysis indentified the possible bacteria candidate possessing the enzyme barbiturase that could degrade pentobarbital [Berryman, 2011].

1.8 Purpose of the Study

Pharmaceuticals have potential adverse human and environmental effects. Pharmaceuticals such as pentobarbital can persist and contaminate the environment. Therefore the effects of pentobarbital in our environment should be studied thoroughly. The determination of pentobarbital in soil is the first step to quantify the amount of pentobarbital in the environment and then examine its degradation pattern.

The purpose of this study is to identify and quantify pentobarbital by liquid chromatography/mass spectrometry coupled to solid phase extraction to determine the breakdown rate of pentobarbital adsorbed in different types of soil. In addition, extraction efficiency and decay rate of pentobarbital were investigated. To facilitate bioremediation a search was conducted for a microbe strain possessing an enzyme capable of breaking down pentobarbital into its metabolites that has leached into soil. Soil types utilized in this study were sand, dirt, potting soil and topsoil.

CHAPTER II

MATERIALS AND PREPARATION

2.1 Materials and Reagents

2.1. a. Bacterial Cell and Soil Samples Used

The Department of Biology at Middle Tennessee State University provided the soil bacterium, alpha small utilized in this study. Alpha small is a gram-negative bacillus and was isolated from soil samples collected from a horse burial mound site in Tennessee. Alpha small is the generic name given for identification purposes only. Initial analysis conducted by the Department of Biology at Middle Tennessee State University via enzyme linked immunosorbent assay (ELISA) and optical density measurements indicated that alpha small was a possible candidate for possessing the enzyme barbiturase that could degrade the barbiturate pentobarbital.

Soil samples such as sand and dirt were obtained from the Department of Biology at Middle Tennessee State University. Department of Agriculture and Environmental Science at Tennessee State University provided topsoil (10-20cm) samples. Expert Gardener All Purpose Potting Soil Mix was purchased from Lowe's Home Improvement Center.

2.1. b. Chemicals and Reagents Used

Pentobarbital sodium salt, ($C_{11}H_{17}N_2NaO_3$) (99% pure) utilized in the study was purchased from Sigma Aldrich Co. Sodium acetate trihydrate (99% pure) was used to make buffer solution was purchased from Sigma Aldrich Co. Dextrose and minimal broth (Davis without dextrose) dehydrated powders used for bacterial growth purposes were purchased

from Becton, Dickinson and Company. Strata-X solid phase extraction units were purchased from Phenomenex Inc. Millex - GV PVDF 0.22µm syringe driven filters were purchased from Millipore Corporation. HPLC grade methanol and acetonitrile (99.9%) were obtained from Fisher Scientific. All other chemicals utilized in the study were reagent grade. Water (18 MΩ) was used in all required steps.

2.1. c. Instruments Used

Pentobarbital in soil samples were analyzed by a Thermo Scientific Dionex Ultimate 3000 LC/MS system. The column used in the LC/MS was a Phenomenex ODS C18 (150 x 4.6 mm) 5 µm HPLC column purchased from Phenomenex Inc. Bacterial growth and protein concentration were measured using a SP-830 spectrophotometer purchased from Barnstead Turner. Steril Elite 16^{TM} Autoclave from Fisher Scientific, Max Q 4000 Incubator from Thermo Scientific and Roto Shake Gene Shaker from Scientific Industry Inc. were used for preparation of soil samples.

2.2 Preparation of Stock Solution and Calibration Curves

In order to quantify pentobarbital concentration in various soil types, reference materials were employed to establish reliable analytical methods. Pentobarbital stock solution of 10 mg/mL was prepared by dissolving 250 mg of pentobarbital sodium salt in 25 mL of methanol and stored at 4°C. Another stock solution of 1 mg/mL was prepared by diluting 500 µL of the stock solution with 4.5 mL of methanol.

Calibration curve was constructed by diluting the appropriate amount of 10 mg/mL pentobarbital stock solution. Concentrations ranging from 0.001 mg/mL to 0.1 mg/mL were

analyzed using LC/MS. The calibration curve was constructed by plotting the readings of area of the peak from LC/MS against the concentrations of pentobarbital solutions. The coefficient of determination value was utilized to verify the accuracy of the calibration curve. A fresh standard calibration curve was prepared from the stock solution monthly to ensure integrity of the standards. Standards were compared frequently to verify the accuracy of the calibration curve. A new stock solution of pentobarbital in methanol was prepared every three months.

2.3 Soil Sample Preparations and Handling

Soil samples were collected from the Department of Biology at Middle Tennessee State University, the Department of Agriculture and Environmental Sciences at Tennessee State University and from Lowe's Home Improvement Center. Soil mixtures utilized in the study were sand, potting soil, dirt and topsoil (11-20 cm). Topsoil samples (11-20 cm) and dirt were ground with mortar and pestle for more uniform particle size. Since sand and potting soil samples are uniform in particle size, the grinding step was eliminated.

To prepare the soil samples with the desired concentration of pentobarbital, a working solution of pentobarbital was made from a 10 mg/mL stock solution of pentobarbital. The appropriate amount of the stock solution was diluted with methanol to create the working solution.

Long-term soil studies were prepared using three kinds of samples containing 15 grams of potting soil, sand and topsoil (11-20 cm). Each sample was spiked with a known amount of pentobarbital and mixed thoroughly. The soil samples were each divided into three

5 g samples and stored in a 50 mL centrifuge tube and incubated at 37 ºC over a 3-week period of sampling.

Potting soil, sand and topsoil (10-20 cm) were used for autoclaved long term analysis. The soils were sterilized by autoclaving at 121°C for 45 minutes. The soils were then spiked with appropriate amount pentobarbital and mixed thoroughly. The mixed samples were each divided into three 5 g samples and stored in a 50 mL centrifuge tube and incubated at 37 ºC over a 3-week period. Pentobarbital was extracted from the soil and analyzed every week according to the method described in Sections 2.4 and 2.5.

2.4 Extraction of Pentobarbital from Soil Samples

To prepare pentobarbital from soil samples, 25 mL of laboratory grade methanol was added and mixed thoroughly by vortexing and automated shaking overnight. The samples were allowed to settle by centrifuging the samples at 4000 rpm at 4°C (figure 7) and the supernatant was transferred into a 50 mL disposable centrifuge tube. An additional 10 mL of methanol was added to the soil samples, and mixed thoroughly by shaking and vortexing for two hours and centrifuged. The supernatant was removed and combined with the original methanol. The combined methanol solution was centrifuged at 4000 rpm for 30 minutes to remove particulate matter (figure 8). The supernatant was transferred to a round bottom flask to evaporate to near dryness on a rotary evaporator at 37 ºC. The extracts were re-dissolved using 10 mL of 18Ω water. The solution was then filtered through a Millex – GV PVDF 0.22 µm syringe driven filter to remove any particulate matter. The filtrate was then ready for solid phase extraction (Figure 9).

2.5 Solid Phase Extraction (SPE) and Method Verification

Solid phase extraction method utilized Strata-X 33µm polymeric reverse phase extraction cartridges. The cartridges were first activated and conditioned using 2 mL of 5% methanol, followed by 2 mL of 0.1M sodium acetate buffer (pH 7.02). The pentobarbital filtrate sample was loaded onto the cartridge at a rate of 4 - 6 drops per 15 seconds. After pentobarbital sample was loaded, the cartridge was washed with 1 mL of 0.1 M sodium acetate buffer and allowed to dry under vacuum for a minimum of 5 minutes. The last step of the extraction process was the elution of pentobarbital sample from the strata-X cartridge into a fresh vial using 1 mL of 50:50 20% methanol/acetonitrile. The solution was then transferred to an HPLC vial for LC/MS analysis (Figure 9).

Figure 7: Combined 35 mL of methanol solution was centrifuged at 4000 rpm for 30 minutes to remove particulate matter.

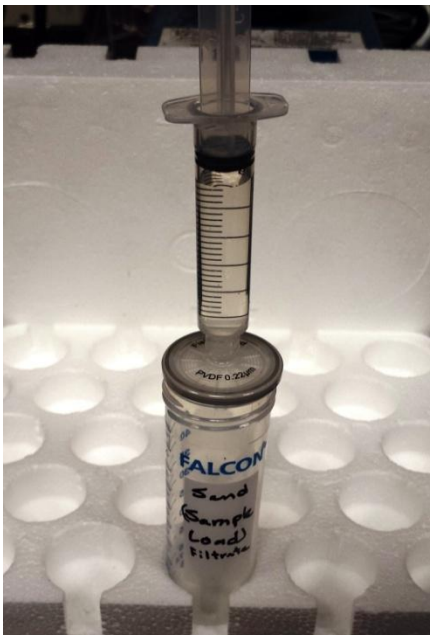


Figure 8: Filtration through a Millex – GV PVDF 0.22 µm syringe driven filter to remove any particulate matter.

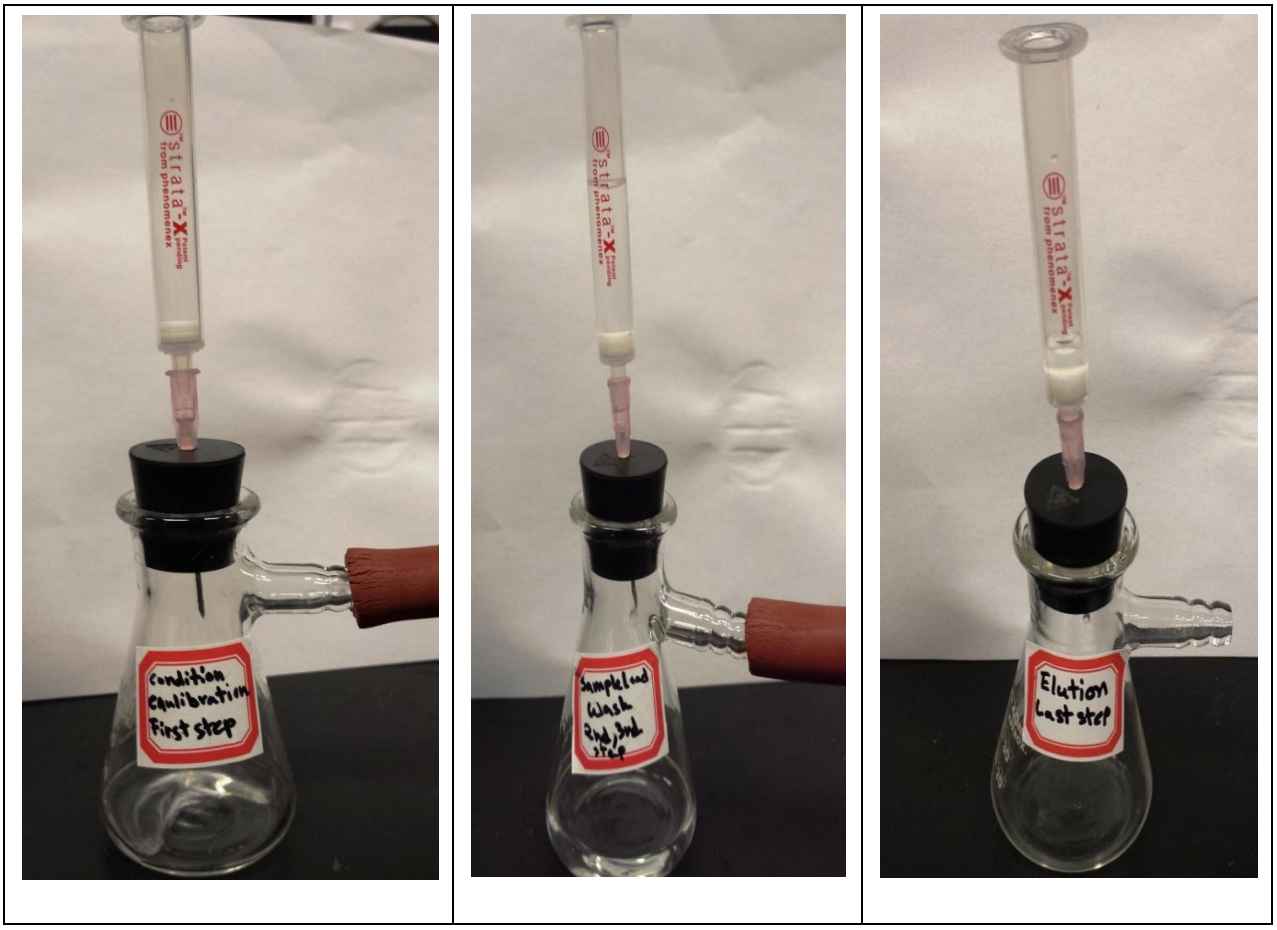


Figure 9: Solid phase extraction process in steps utilizing Strata-X 33µm polymeric reverse phase extraction cartridges.

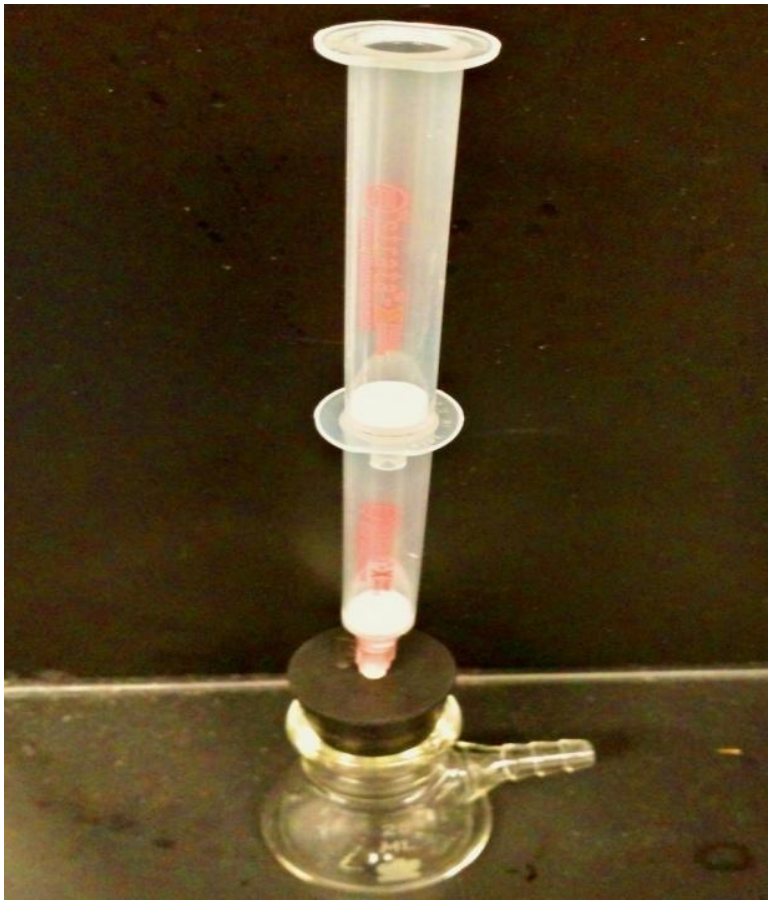


Figure 10: A second SPE cartridge was attached to determine if analyte breaks through the first SPE cartridge during the adsorption phase.

2.6 Liquid Chromatography / Mass Spectrometer (LC/MS) Method

The amounts of pentobarbital in samples were determined by LC/MS. The samples were injected onto Phenomenex ODS C18 150 x 4.6 mm 5 µm reverse phase column eluted with acetonitrile:water (60:40). The method parameters are detailed in the results and discussion section. The sample was eluted by a isocratic elution with more than 10 minutes injection to insure no cross contamination between the samples. Pentobarbital retention time was 3.4 minutes for both standards and samples. The ion spectrum for pentobarbital [M-H]- is 225m/z.

2.7 pH Measurement of Soil Samples

The pH of each soil sample was measured to understand the surface interaction between the different soils and the pentobarbital solution. Ten grams of soil sample was weighed and 90 mL of 18Ω water was added to make a 10% solution of the soil sample. Using a calibrated pH meter, pHs were measured for potting soil, sand, topsoil (10-20cm) and dirt.

2.8 Minimal Broth Preparation

The culture medium for bacterial growth used in the study was formulated using BD Difco™ Dehydrated Culture Media: Minimal Agar Davis. Minimal broth was prepared using 10.6 grams of the powder in 1 liter of deionized water. The solution was heated with frequent stir and boiled for 1 minute to completely dissolve the powder. The mixture was divided into four 50 mL and four 500 mL of Erlenmeyer flasks. The Erlenmeyer flasks were autoclaved at 121°C and the final pH of the mixture was 7.1.

2.9 Bacteria Preparation

The bacterium utilized in this study was alpha small cells. The alpha small cells were previously suspended in 10 mL of 20 mM potassium phosphate buffer solution at 4°C. Bacteria samples were cultured in 25 mL of the minimal solution and 50 µL of the bacterial cells in 20 mM potassium phosphate buffer solution . The 25 mL samples were placed in an incubator at 37 ºC and 181 rpm for 24 hours. Using a spectrometer at 600 nm, blanked using 18Ω water, 1 mL samples were analyzed hourly for bacteria growth. After forty eight hours, at optical density around 0.6, bacteria samples were removed from the incubator and centrifuged at 10,000 x G for 15 minutes. The supernatant was stored for determining the pentobarbital concentration using the method described in section 2.11developed previously by Bagsby. The bacteria cells were resuspended in 0.5 mL of sterile 20 mM potassium phosphate buffer (pH 7.0), and sterile glycerol and stored at -80 ºC.

2.10 Analysis of Barbiturase Activity (Kinetic Activity)

Alpha small cells were suspended in 30 mL of 20 mM potassium phosphate buffer solution (pH 7.0). Cells were broken in 10-second intervals with sonication at 4 ºC for 20 minutes. The solution was then centrifuged at 10,000 RCF for 25 minutes at 4ºC to eliminate the cellular debris. The supernatant was collected and analyzed using the Bio-Rad protein assay to confirm the presence of protein.

The protein present in the supernatant was utilized to see if it degrades pentobarbital. Two vials labeled A and B containing pentobarbital and water were used for this part of study. Vial A contained 10 mL water, 0.4 mL of 1 mg/mL pentobarbital, 100 µL initial extract recovered from alpha small bacteria after sonication. Vial B contained 10 mL water,

0.4 mL of 1 mg/mL pentobarbital and no initial extract. Representative samples from each vial were collected at designated intervals to analyze if there was any degradation of pentobarbital.

2.11 Alpha Small Bacteria Growth in Glucose and Pentobarbital

Six flasks containing 50 mL of minimal broth for alpha small bacterial culture were prepared containing increasing percentage of pentobarbital and decreasing percentage of glucose. Bacterial growth was monitored in a spectrometer at 600 nm. Samples were collected at designated time intervals to determine if there was any degradation of pentobarbital in the minimal broth as the bacteria grew. All the samples were processed through solid phase extraction before analyzing using LC/MS.

CHAPTER III

RESULTS AND DISCUSSION

3.1 LC/MS Sensitivity, Optimization and Limits of Detection

Pentobarbital was quantified by a method developed by a previous group member involving solid phase extraction and LC/MS [Bagsby, 2013]. The conditions used in this study followed the previously developed method, which produced reliable and reproducible data. A Phenomenex ODS C18 (150 x 4.6 mm) 5 µm reverse phase column was used at 40°C. Mobile phase was composed of acetonitrile:water (60:40). The sample was eluted by a isocratic elution with more than 10 minutes injection to insure no cross contamination between the samples The retention time for pentobarbital was approximately 3.4 minutes for both standards and samples. The ion spectrum, utilizing peak at negative polarity, for pentobarbital [M-H]- was 225m/z. The method parameters are detailed in Table 2.

Table 2: LC/MS parameters established for detection of pentobarbital

LC/MS Method Parameters	
Column	ODS C-18 (150 x 4.6 mm)
Elution	Isocratic
Mobile Phase	Acetonitrile and 18Ω Water
Mobile Phase Ratio	60/40
Injection Volume	10µL
Flow Rate	0.6 mL/min
Ionization Mode	ES-API
Fragmentation Voltage	90
Column Temperature	40°C
Retention Time	3.4 minutes
$[M-H]^-$	225 m/z
Polarity	Negative
Mode	SIM

Figure 11 is the total ion chromatogram of pentobarbital standard and pentobarbital recovered from sand sample. Pentobarbital was detected at 225m/z using the established LC/MS parameters (Table 2). The retention time for pentobarbital was approximately 3.4 minutes.

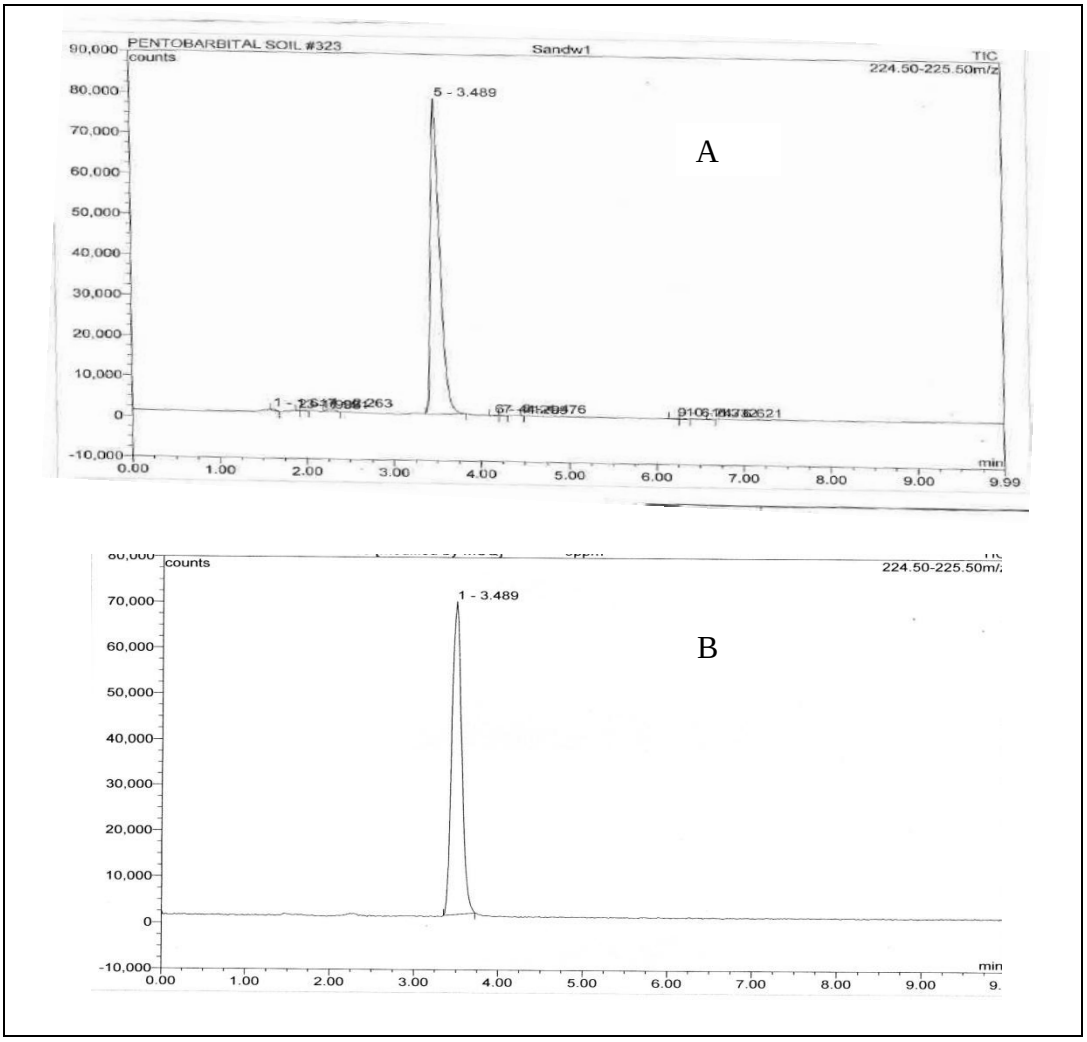


Figure 11: A: Total Ion Chromatogram (TIC) of pentobarbital. B: is the total ion chromatogram of pentobarbital standard sample.

Figure 12 is the mass spectrum of the ions present in the previous peak (Figure 11). The detection method was effective at indentifying pentobarbital at 3.4 min. The mass to charge ratio (m/z) of pentobarbital [M-H]- in negative mode is 225 and is the strongest peak in the spectrum.

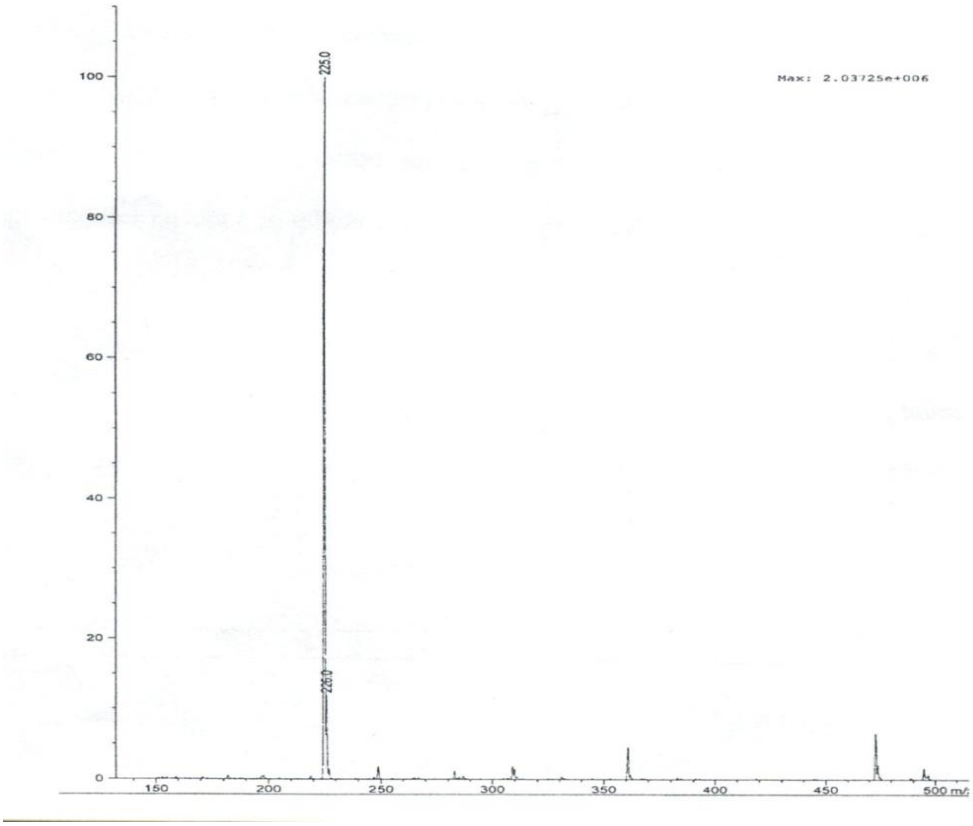


Figure 12: Ion spectra of pentobarbital in sand sample analyzed via LC/MS utilizing the method parameter detailed in Table 2. Pentobarbital has a mass to charge ratio (m/z) of 225 in negative polarity mode.

Limit of detection (LOD) was established in the mass spectrometer using the same method. Limit of detection is the lowest concentration or quantity of a component that can be detected with established analytical method [Armbruster, 2008]. Limit of quantification (LOQ) is the smallest concentration of a component that can be reliably detected with established analytical method. Limit of quantification can be equivalent or much higher concentration than limit of detection [Armbruster, 2008].

To establish a calibration curve, a series of pentobarbital solutions ranging from 10 mg/mL (10,000 ppm) to 0.0001 mg/mL (0.1 ppm) were utilized. The LC/MS was capable of detecting 0.0001 mg/mL (0.1 ppm) using the established method which is three times higher than the noise level (Figure 13B). However, the limit of quantification occurred at 0.0005 mg/mL (0.5 ppm) (Figure 13A). The upper limit for quantifying pentobarbital was 0.10 mg/mL (100 ppm). Above 0.10 mg/mL (100 ppm) calibration curve deviated from linearity. Therefore, LC/MS was suitable for the analysis of pentobarbital in the range of 0.0005 mg/mL (0.5 ppm) to 0.10 mg/mL (100 ppm).

The following figures are the chromatograms of the pentobarbital in methanol solution demonstrating the peak intensity and lower limit of detection (LOD).

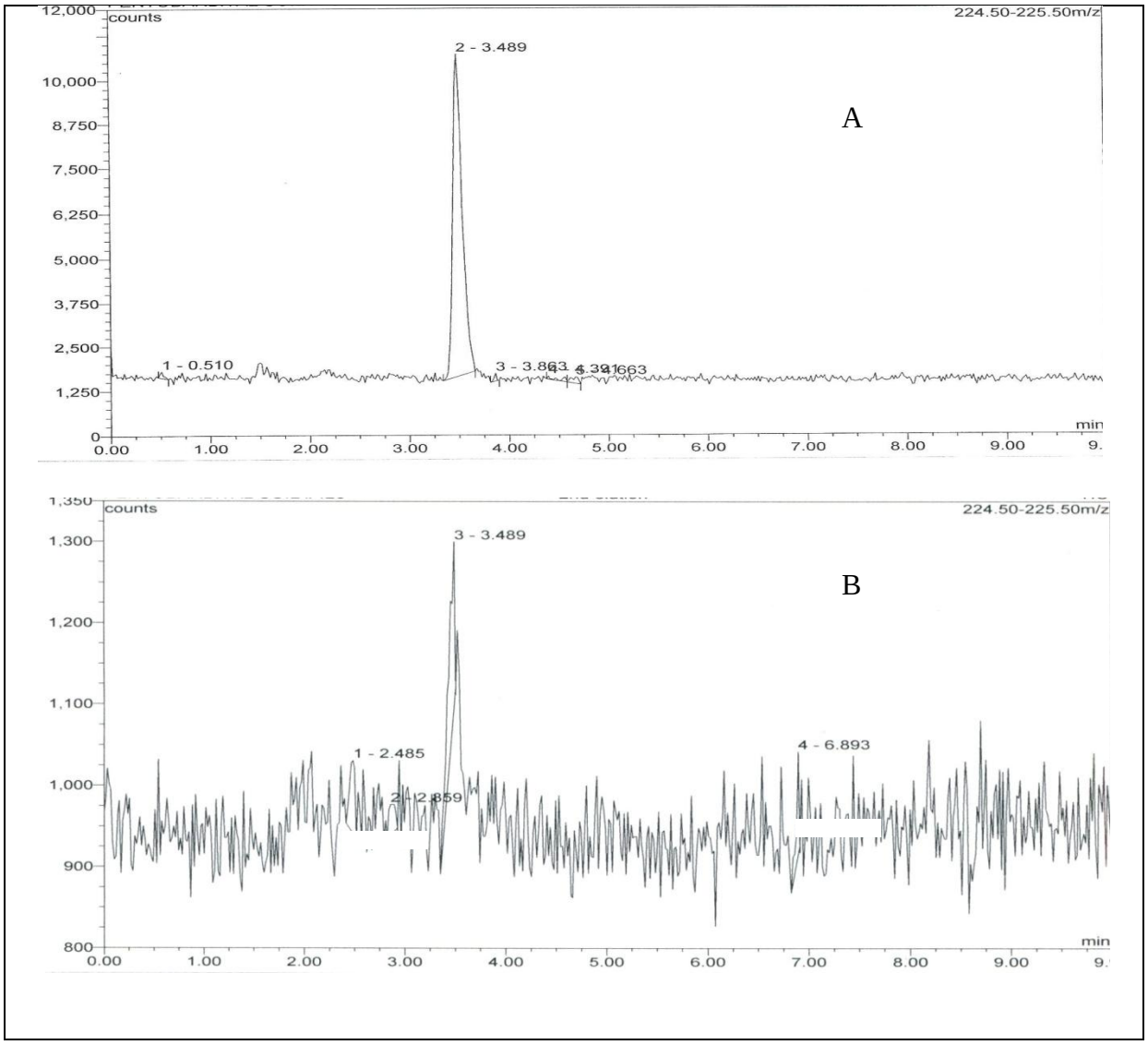


Figure 13: A: Limit of quantification occurred at 0.0005 mg/mL (0.5 ppm) in LC/MS using the established method. B: Limit of detection (LOD) occurred at 0.0001 mg/mL (0.1 ppm) in LC/MS using the established method.

3.2 Calibration Curve

The following figure shows the calibration curve established using the method to determine the upper and lower range of sensitivity of the instrument (Figure 14). The instrument was calibrated frequently and the updated calibration curve was used for analysis. The preparation of the standards using pentobarbital and methanol were described in section 2.2.

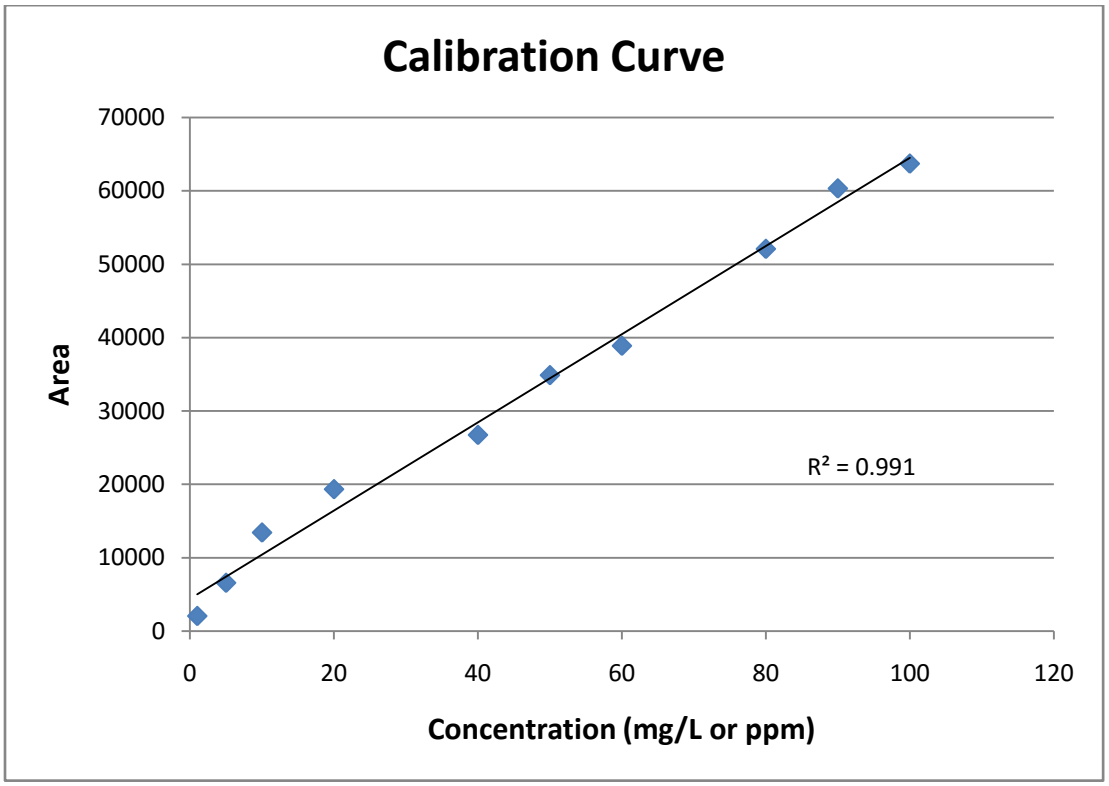


Figure 14: Calibration curve established for pentobarbital in methanol as detected by liquid chromatography / mass spectrometry. The graph illustrates the relationship between the area of the peak for pentobarbital and the concentration of pentobarbital.

3.3 Solid Phase Extraction Method Verification

Solid phase extraction method utilized Strata-X 33µm polymeric reverse phase extraction cartridges. Solid phase extraction is an easy cleanup process for complex matrices such as soil. Three possible scenarios were studied to confirm the efficiency of the solid phase extraction method as described in Chapter II section 2.5. Figures 15 and 16 confirm the efficiency of the solid phase extraction method.

(1) A second SPE cartridge was placed underneath the original cartridge to determine if the analyte breaks through the first SPE cartridge during the adsorption phase (Figure 15A). The cartridge placed underneath was treated the same way as the first cartridge and eluted samples were analyzed by LC/MS. The peak area from the second SPE cartridge was below the limit of quantification.

(2) The extraction after washing step was analyzed using LC/MS to determine if analyte partially breaks through during the washing step. Figure 15B shows no pentobarbital in the washing step.

(3) The last step of solid phase extraction was elution of the pentobarbital sample from the Strata-X cartridge into a fresh vial using 1 mL of 50:50 20% methanol/acetonitrile. The cartridge was eluted three times. The last two elutions labeled second and third elution were analyzed by LC/MS. This was conducted to test if the first elution using 1 mL of 50:50 20% methanol/acetonitrile was sufficient to elute pentobarbital bound to the cartridge. Figures 16 A, B and C show that the first elution with 1 mL of 50:50 20% methanol/acetonitrile was sufficient to elute the pentobarbital from the solid phase extraction

cartridge. Negligible amounts of pentobarbital were recovered during second and third elutions.

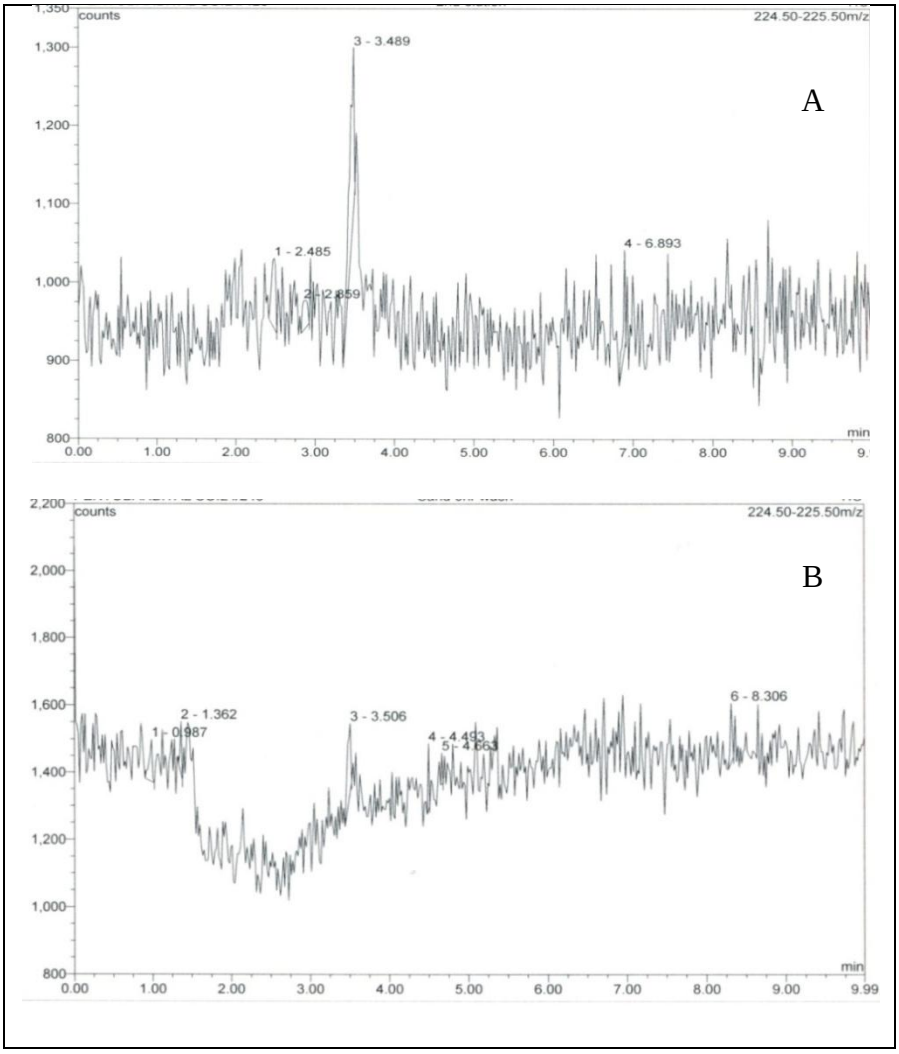


Figure 15: A**:** Second SPE cartridge was placed to detemine if analyte breaks through the first SPE cartridge during the adsorption phase. B: The extraction after washing step was analyzed using LC/MS to verify if analyte partially breaks through in washing step.

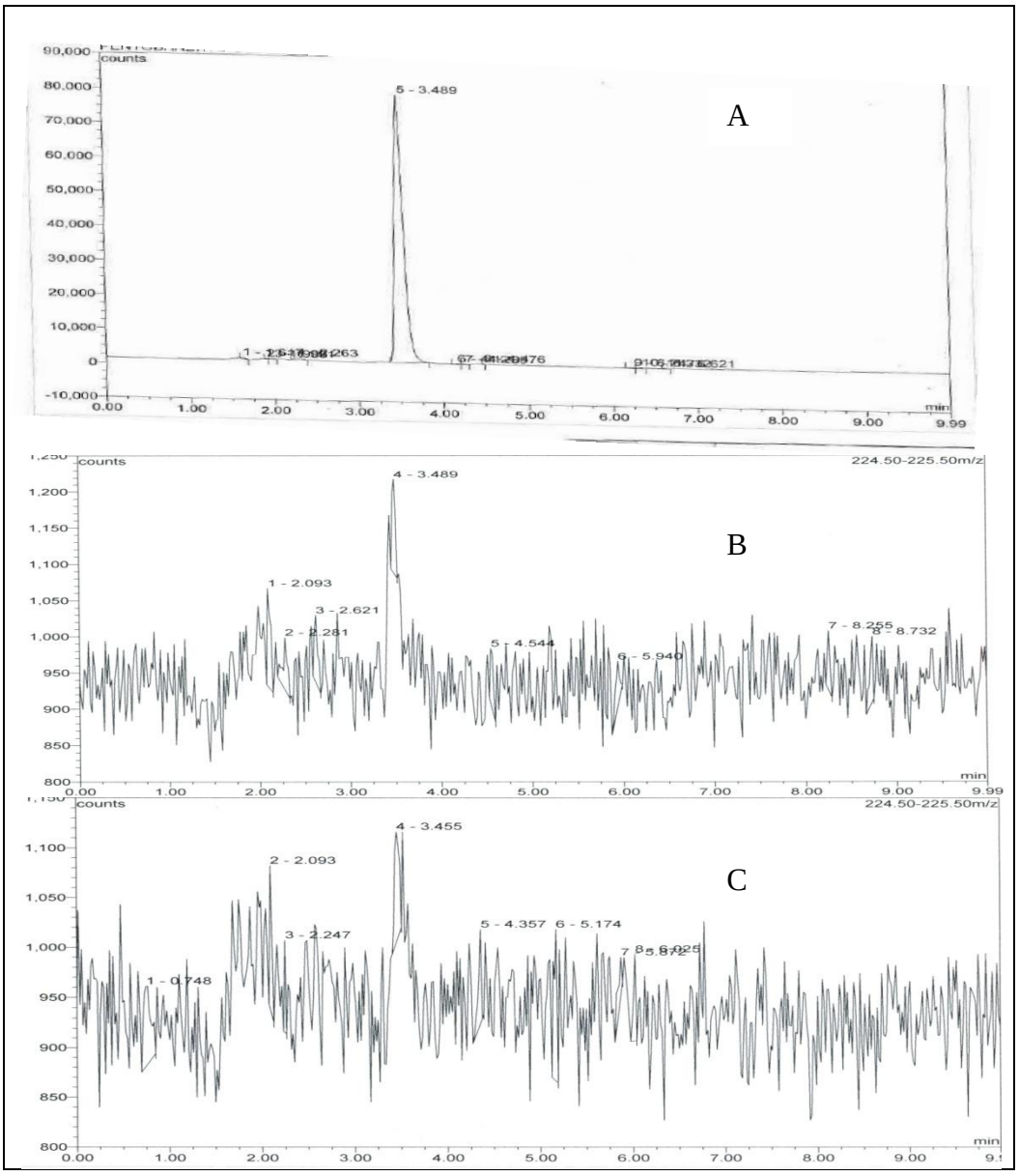


Figure 16: A**:** The elution step chromatogram indicates that the first elution with 1 mL of 50:50 20% methanol/acetonitrile was sufficient to elute the pentobarbital from the solid phase extraction cartridge. B: The second elution step with 1 mL of 50:50 20% methanol/acetonitrile. C: The third elution step with 1 mL of 50:50 20% methanol/acetonitrile.

3.4 Recovery of Pentobarbital from Various Soil Types

Based on the developed method, soil samples needed to contain between 0.0005 mg/mL (0.5 ppm) to 0.10 mg/mL (100 ppm) pentobarbital to quantify by LC-MS after the extraction process. Therefore a concentration was selected which falls in the limit of quantification on the LC/MS and has less possibility to be lost by the extraction and detection process.

A series of experiments from day 0 to 4 on various soil types were conducted to observe the degradation pattern of pentobarbital and the extraction efficiency. A standard calibration curve was generated before analyzing soil samples to ensure reproducibility. Approximately 25 grams of soil samples were spiked with 0.2 mg of pentobarbital (8 µg pentobarbital per gram of soil) and mixed thoroughly using vortex and auto-shaker. Following the preparation of the spiked samples, the soils were divided into 5 gram samples and stored in 50 mL centrifuge tubes and incubated at 37ºC for daily sampling. The 0 hr sample for daily soil analysis was not incubated.

Extraction of pentobarbital was performed by adding 25 mL of reagent grade methanol to the soil samples and mixing them overnight by shaking followed by vortexing. The methanol layer was removed and an additional 10 mL of methanol was added to the soil samples and mixed for two hours. The methanol layers were combined and centrifuged to remove any debris. The supernatant was evaporated to near dryness using a rotary evaporator. Then the sample was re-dissolved using 10 mL of 18Ω water. The solution was filtered through a 0.22 µm syringe driven filter and the filtrate was subjected to solid phase

extraction followed by LC/MS analysis. The results of the method with pentobarbital are detailed in Table 3

Table 3: Daily percent recovery of pentobarbital from various soil types.

Soil type	Day	PB Injected in per Experiment (mg)[a]	PB Recovered in per Experiment (mg)	Percent Recovery[b] (%)
Sand	0	0.040	0.044	111
	1	0.037	0.034	92
	2	0.040	0.039	99
	3	0.040	0.042	107
	4	0.041	0.035	87
Dirt	0	0.040	0.044	110
	1	0.040	0.040	100
	2	0.040	0.026	66
	3	0.040	0.030	75
	4	0.041	0.037	91
Potting Soil	0	0.040	0.037	92
	1	0.040	0.035	88
	2	0.041	0.036	89
	3	0.040	0.025	63
	4	0.041	0.025	63

[a]Pentobarbital injected per experiment (mg) is the calculated amount of pentobarbital in the soil sample determined from the stock solution.
[b]Percent recovery was the percentage of pentobarbital (mg) recovered from the soil sample as measured by LC/MS versus the amount of pentobarbital added to the sample.

The daily analysis shows recoveries at zero hour were about 100% for sand and dirt samples. The recoveries of potting soil declined as time passed. By day 4, potting soil recoveries declined significantly to 63%. However, sand samples recoveries stayed high for the first 4 days. The recovery of pentobarbital from sand sample declined to 87% by the fourth day. Similar recoveries of pentobarbital were observed in dirt samples. The recovery of pentobarbital was 110% for 0 hour, which declined to a 91% by day 4.

3.5 Weekly Study of Pentobarbital in Various Soil Types

Previously, degradation of pentobarbital in various soil types was studied from week 4 to week 17 [Bagsby, 2013]. In the current study, the initial three week period of soil analysis was conducted using a similar approach to measure the degradation pattern of pentobarbital in different soil types. The analysis was conducted using three 15-gram samples of potting soil, sand and topsoil (10-20 cm). The soil samples were spiked with 0.03 mg of pentobarbital (2 µg of pentobarbital for 1 gram of soil) and mixed thoroughly using vortex and auto-shaker. Following the preparation of the spiked samples, the soils were divided into 5 g samples, stored in 50 mL centrifuge tubes, and incubated at 37ºC. The extraction method and the analysis were performed according to the procedure described in Section 2.2. The results from the weekly study with pentobarbital are detailed in the following table.

From Table 4, it appears that recovery declined for pentobarbital significantly in potting soil. The long-term recovery of pentobarbital in sand, topsoil and dirt also declined significantly. The study was conducted in such a way that pentobarbital could not be removed by ground water. There is a possibility that pentobarbital was irreversibly sorbed to the soil. However, irreversible sorption to the soil can be discounted for sand and dirt because the extraction recovery at 0 hour was around 100% (Table 3). Although the possibility of pentobarbital irreversibly sorbed to potting soil cannot be discounted (Table 3).

According to the literature, there are a numerous number of microorganisms in soil [USDA, 2013]. Microbial degradation could also be a contributing factor to the degradation of pentobarbital. Finally, the nature of the soil itself could contribute to the breakdown of pentobarbital.

Table 4: Weekly degradation of pentobarbital in various soil types.

Soil Type	Analysis Period (Week)	Percent Recovery (%)
Sand	1	95
	2	83
	3	78
	4*	37
	8*	38
	11*	23
	17*	17
Dirt	1	119
	2	79
	3	58
Top soil	1	80
	2	55
	3	42
	4*	38
	8*	41
	11*	20
	17*	19
Potting soil	1	68
	2	47
	3	37
	4*	34
	8*	30
	11*	10
	17*	10

*Results from week 4 to 17 were conducted previously [Bagsby, 2013].

The three-week period of soil analysis was conducted to be consistent with the previous long-term analysis. Soil samples, amount of pentobarbital and conditions were consistent for the analysis. Over the 3-week period of analysis, the recovery of pentobarbital from potting soil declined significantly. Over the 17-week period of analysis, the recovery declined to 10.0% for potting soil. The long-term recovery of pentobarbital in sand, dirt and topsoil was also compromised. The recovery of pentobarbital in sand declined from 95% to 78% in the first three weeks of analysis. Topsoil had a recovery of 80% for the 1-week sample and a 19.3% recovery for the 17-week sample. Pentobarbital recovery in dirt was not analyzed for the 17-week period. However, it shows a similar trend. Dirt had a recovery of 119% for the 1-week sample and a 58% recovery for the 3-week sample. A pattern of depleting recovery of pentobarbital was consistent for all the soil types.

3.6 Weekly Degradation Study of Pentobarbital in Autoclaved Soils

To determine if any bacteria caused the degradation of pentobarbital in soil samples, all the soil samples were autoclaved at 121°C before spiking with pentobarbital solution. The experimental procedure and the pentobarbital analysis were similar to that described in section 2.

Table 5 shows that pentobarbital concentration did not decline in sand and topsoil samples after autoclaving the soils. This indicates that there might be a microbial interaction responsible for breaking down pentobarbital in sand and topsoil. However, significant degradation was observed in the potting soil. The degradation of pentobarbital in potting soil might be due to several reasons.

Table 5: Weekly degradation of pentobarbital in different autoclaved soils

Sample	Analysis period (Week)	Percent Recovery (%) trial1	Percent recovery (%) Trial 2
Sand	1	109.4	100.7
	2	103.2	105.4
	3	99.6	106.8
Potting Soil	1	94.6	93.2
	2	76.4	72.6
	3	35.3	45.1
Top Soil (10-20 cm)	1	108.3	104.8
	2	104.4	107.1
	3	105.2	101.5

Potting soil utilized in this study contains peat moss, composted bark, pasteurized poultry litter, and an organic wetting agent. With no autoclaving, bacterial growth in the potting mix during the incubation is a possibility since the potting soil is composed of many materials. There is a chance that the substances of potting soil are causing pentobarbital degradation. While it may not be the only contributor to the break down, it could be a significant one, because declined recovery of pentobarbital was observed after the soils was autoclaved. Figure 17 shows the chromatograms of pentobarbital from autoclaved soils analyzed via LC/MS using the established method.

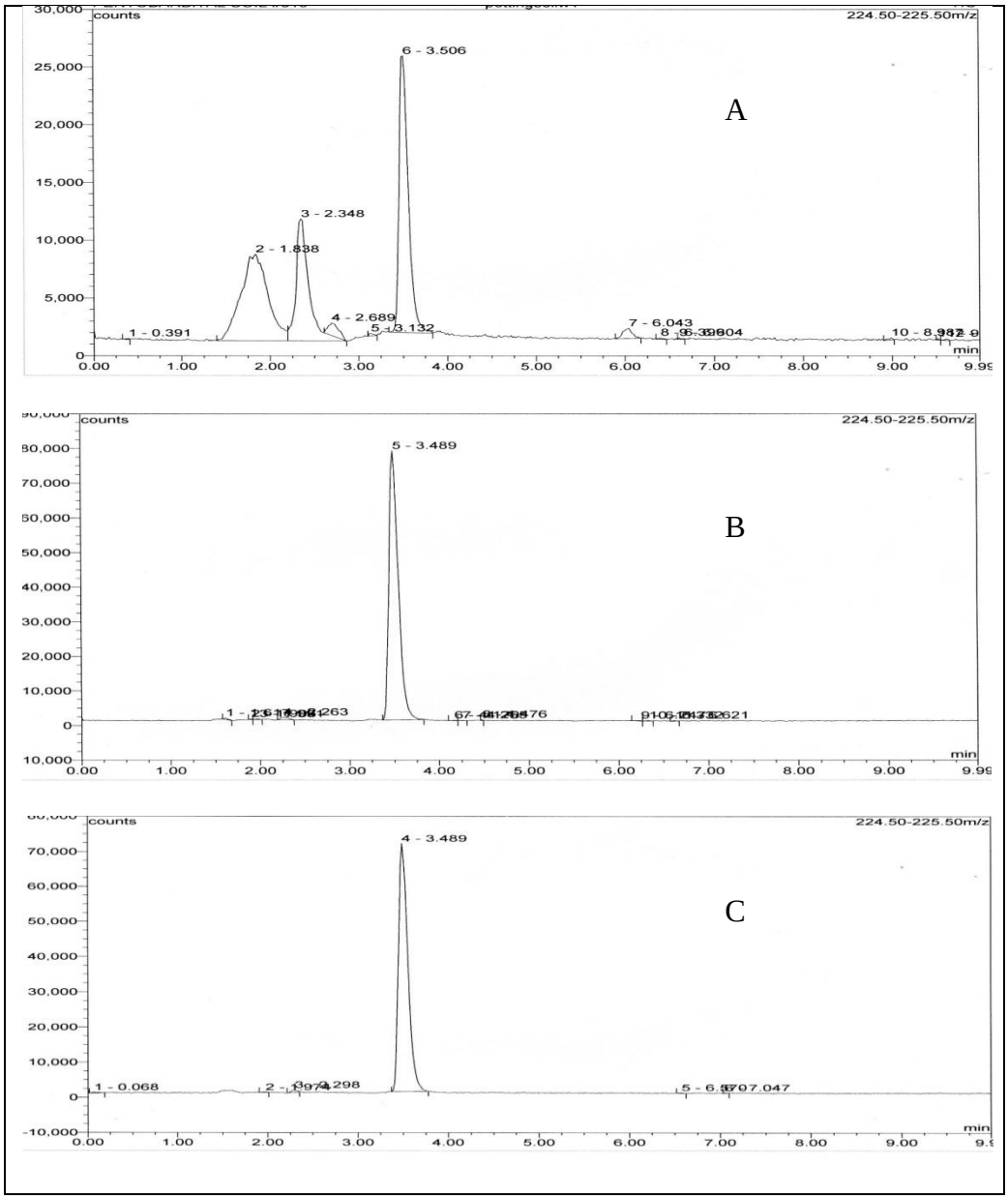


Figure 17: Chromatograms of pentobarbital from autoclaved soils analyzed via LC/MS using the established method. A: Potting Soil B: Sand C: Topsoil (10 - 20 cm)

3.7 Pentobarbital Breakdown by Bacteria and Enzyme Analysis

The analysis conducted by the Department of Biology at Middle Tennessee State University via enzyme linked immunosorbent assay (ELISA) and optical density measurements indicated that alpha small bacteria was a possible candidate for possessing the enzyme barbiturase that could degrade pentobarbital. Alpha small is a gram negative, rod shaped soil bacteria. Previous experiments illustrated that alpha small bacteria has the possibility to contain an enzyme which is capable of metabolizing pentobarbital.

To determine if alpha small bacteria contains the enzyme to degrade pentobarbital further analysis was conducted. Alpha small cells were grown to an optical density of 0.651 at 600 nm and were suspended in 30 mL of 20 mM potassium phosphate buffer solution (pH 7.0). Cells were disrupted with sonication and the solution was centrifuged to eliminate any cellular debris. The supernatant was collected and was analyzed for protein content using the Bio-Rad protein assay to confirm the disuption of the cells. Figure 18 shows the Bio-Rad protein assay standard curve. Table 5 shows the positive results for presence of protein in supernatant. The average protein concentration was 0.109 ± 0.049 μg/μL.

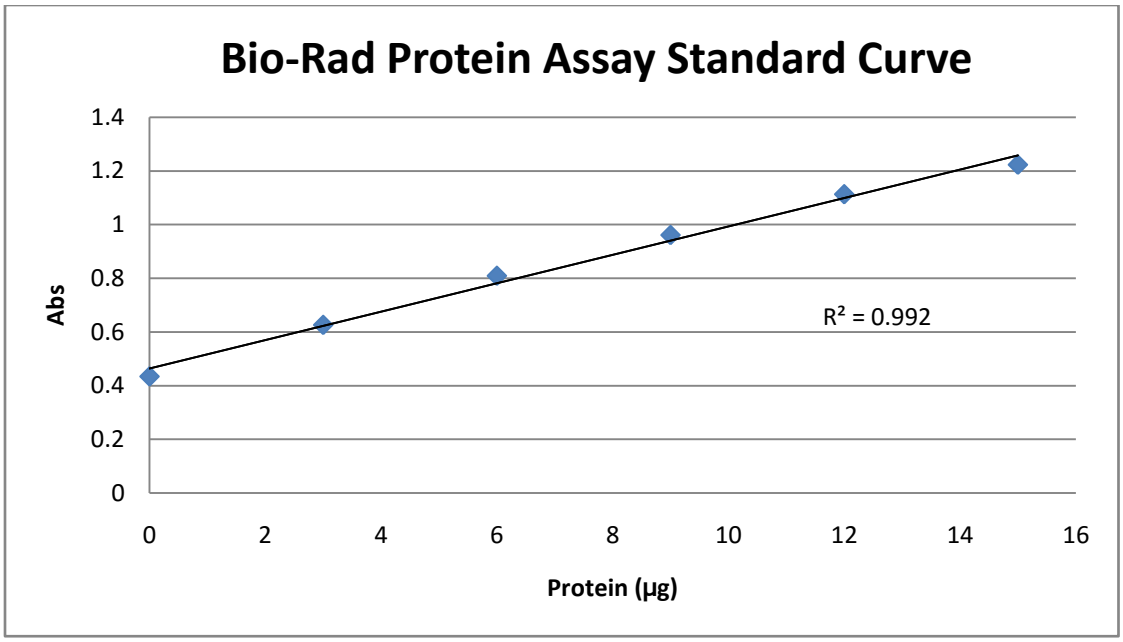


Figure 18: Bio-Rad Protein Assay standard curve. This curve was used to confirm the amount of protein found in the supernatant after sonicating the alpha small bacteria.

Table 6: Initial extract recovered from alpha small bacteria in the supernatant after sonification.

Amount of initial extracts, (µL)	Absorbance	Amount of protein (µg)
10	0.491	0.52
20	0.608	2.8
50	0.811	6.7

The protein present in the supernatant was analyzed to see if it degrades pentobarbital. Two vials, A and B containing pentobarbital and water were used for this part of the study. Vial A contained 10 mL water, 0.4 mL of 1 mg/mL pentobarbital, 100 µL initial extract recovered from alpha small bacteria after sonification. Vial B contains 10 mL water, 0.4 mL of 1 mg/mL pentobarbital and no initial extract. Representative samples from each vial were collected at designated time intervals to determine if there was any degradation of pentobarbital occurs. All the samples were processed through solid phase extraction before analyzing using LC/MS.

Table 7: Initial extract from alpha small bacteria to determine if pentobarbital was degraded.

Time (hr)	**Peak area (Vial A)**	**Concentration of Pentobarbital (ppm)**	**Peak area (Vial B)**	**Concentration of Pentobarbital (ppm)**
0	25186	35	28966	42
2	24497	34	28105	40
4	23669	33	26913	38
6	24340	34	26162	37
24	23789	33	23791	33
48	21474	29	25333	36
96	22953	32	24117	34

Table 7 shows the breakdown of pentobarbital in the presence of an initial extract from the alpha small bacteria. The actual initial pentobarbital concentration in vial A and B was 40 ppm. The results are negative for activity. As time elapsed, there was no significant decrease in the concentration of pentobarbital in vial A, which contains the initial extract from the alpha small bacteria.

3.8 Alpha Small Bacteria Growth in Glucose and Pentobarbital

The alpha small bacteria grown in pentobarbital and glucose were also studied to see if the bacteria consume pentobarbital as it grows in culture media. Bacterial growth was monitored in a spectrometer (SP-830) at 600 nm. Table 8 shows bacterial growth in pentobarbital and glucose. Six minimal broths for bacterial culture were prepared containing increasing percentage of pentobarbital and decreasing percentage of glucose. Table 8 shows no bacterial formation in Samples 5 and 6. Both sample 5 and 6 contained high amount of pentobarbital and no glucose. Therefore, alpha small does not utilize pentobarbital as a source of carbon or energy.

Table 8: Bacterial growth in minimal broth containing increasing percentage of pentobarbital and decreasing percentage of glucose.

Sample no	Minimal broth (mL)	Percent glucose (%)	Percent pentobarbital (%)	mL of Bacteria Inoculated	Bacteria Growth
1	50	0.20	0.00	2	Yes
2		0.15	0.05		Yes
3		0.10	0.10		Yes
4		0.05	0.15		Yes
5		0.00	0.15		No
6		0.00	0.20		No

From the above table, samples 2, 3 and 4 were studied for 24 hours to see if the growing bacteria consume pentobarbital supplementing the minimal broth. Bacterial growth was monitored in a spectrometer (SP-830) at 600 nm. Samples were collected in designated time intervals to determine if there was any degradation of pentobarbital in the minimal broth as the bacteria grew. All the samples were processed through solid phase extraction before analyzing using LC/MS.

Table 9: Bacterial sample analyzed during the growth phase using LC/MS.

Sample			**Hour**	**Concentration of Pentobarbital (ppm)**
2	**0.15% Glucose**	**0.05% PB**	Initial	443
			2	464
			4	336
			24	356
3	**0.1% Glucose**	**0.1% PB**	Initial	981
			2	933
			6	931
			24	854
4	**0.05% Glucose**	**0.15% PB**	Initial	1575
			20	1348
			24	1278
			43	1222

Sample 2, 3 and 4 in Table 9 shows decrease in concentration of pentobarbital with the growth of alpha small bacteria. Sample 4, which has the highest concentration of pentobarbital, shows a 22% decrease in the concentration after 43 hours. There was a 13% decrease in Sample 3 and 20% decrease in Sample 2. However, no growths of alpha small bacteria were observed in Samples 1, 5 and 6 (Table 8) which were not supplemented with glucose. Although the drops in pentobarbital concentration were not significant, it indicates that, the alpha small bacteria were using the pentobarbital as they grow. Pentobarbital may not serve as a carbon source for the bacteria. However, the alpha small bacteria appear to breakdown pentobarbital if supplemented with glucose.

CHAPTER IV

CONCLUSION

Increasing usage of pharmaceuticals is one of the major factors for contaminating the environment. Pharmaceuticals that persist in the environment have potential adverse human and environmental effects. Pharmaceuticals such as pentobarbital that persist in the environment tend to leach into soil, water and food supply. Therefore, it is important to develop a method to detect and study the degradation pattern of pentobarbital in complex matrices. In this study, degradation of pentobarbital in various soil types was observed. In addition, microbial degradation of pentobarbital was explored.

Solid phase extraction coupled with LC/MS was an efficient method for detecting and quantifying pentobarbital from complex matrices, such as soil. The established method was capable of quantifying pentobarbital in soil at a concentration of 0.5 µg of pentobarbital per gram of soil.

Long-term and daily analysis of various soil samples shows that, the percent recovery of pentobarbital declined slowly in sand, dirt, and topsoil. However, in potting soil the percent recovery of pentobarbital decreased more rapidly. In week 1, the percent recovery of pentobarbital in potting soil was 68%. To observe the microbial influences, soil samples were autoclaved at 121°C to eliminate any possible bacterial growth. The recovery rate of pentobarbital indicates that there might be a microbial interaction responsible for breaking down pentobarbital in sand and topsoil. However, in potting soil breakdown of pentobarbital was observed after autoclaving the soil might be due to the soil characteristics. While it may

not be the only contributor to the breakdown pentobarbital, it could be a significant one. The daily, long-term and autoclaved soil sample study suggests that, the degradation of pentobarbital can be due to microbial influences or nature of the soil or possibly both.

Analysis of the degradation of pentobarbital using a soil bacterium was also a part of the study. Previous studies and preliminary ELISA analysis suggested that alpha small, a gram-negative soil bacteria has the possibility to contain an enzyme that is capable of metabolizing pentobarbital [Bagsby, 2013]. Further analysis of alpha small bacterial cell extracts confirmed the presence of protein in the supernatant. However, the protein present in the supernatant was not capable of degrading pentobarbital over time. The original concentration of pentobarbital (40 ppm) did not decrease significantly after initial extracted protein was employed. Possible that there is a membrane - associated barbiturase.

The alpha small bacteria growth in pentobarbital and glucose were also studied to observe if the bacteria consume pentobarbital as it grows in culture media. Alpha small bacteria grew only in the culture media that were supplemented by glucose and pentobarbital. Bacterial growth was not observed in the pentobarbital-supplemented culture media without glucose. Slight decay (about 18%) in pentobarbital concentration was observed in the minimal broth culture media containing glucose and pentobarbital. Although the drops in pentobarbital concentration were not significant, it indicates that, the alpha small bacteria were using the pentobarbital as they grow. However, pentobarbital may not serve as a carbon or nitrogen source for the bacteria. It is also possible that if the enzymes responsible for pentobarbital breakdown are carried on a extra chromosomal plasmids or intra chromosomal transposons. During "routine" culture in media, the bacteria may not need the extra DNA or

selectively lose those genes. Therefore, the degradation of pentobarbital through the presence of alpha small bacteria or barbiturase were inconclusive. The bacterium may contain the enzyme capable of metabolizing pentobarbital however further research is necessary.

Research in the area of pentobarbital stability and decay is currently under investigation. Long term, daily, and autoclaving recovery data suggests that pentobarbital may not be as stable as expected in all matrices, especially in potting soil samples. Further investigation of potting soil constituents will help to understand the degradation pattern of pentobarbital. Additional research needs to be conducted using alpha small bacteria for longer period to understand the pentobarbital degradation pattern. In addition, a search for bacteria in the soil samples can be performed which are capable of metabolizing barbiturates. A study can be conducted to observe if mutagenize alpha small cells have selective growth on pentobarbital. Furthermore, a search can be performed for organisms that synthesize pentobarbital, effective against predators.

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