EXTRACTION AND DETECTION OF PENTOBARBITAL IN SOIL BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY / MASS SPECTROMETRY

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ABSTRACT

EXTRACTION AND DETECTION OF PENTOBARBITAL IN SOIL BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY / MASS SPECTROMETRY

Chasity Bagsby

A method for detecting the pharmaceutical drug, pentobarbital, in the complex matrix soil has been developed and will be detailed in this thesis. Of growing concern is the release of pharmaceuticals in the environment. Pentobarbital is an organic compound in the barbiturate family that is used often in the euthanasia of animals. Once these animals are buried, pentobarbital may leach into the surrounding soil and become a source of contamination. Satisfactory recoveries of the pentobarbital from soil indicate LC/MS coupled with solid phase extraction is an effective method for analysis and detection. Pre-concentration via solid phase extraction allowed 0.001 mg of pentobarbital per 5 grams of soil (200 ppb) to be detectable at limits of quantification using liquid chromatography/mass spectrometry. This method is suitable for larger quantities of soil and applicable for a wide range of soil types.

This method has further applications in determining the decay and dispersion of pentobarbital in soil. Other applications include studying soil bacterium that possess an enzyme reported to be capable of breaking down pentobarbital into its metabolites.

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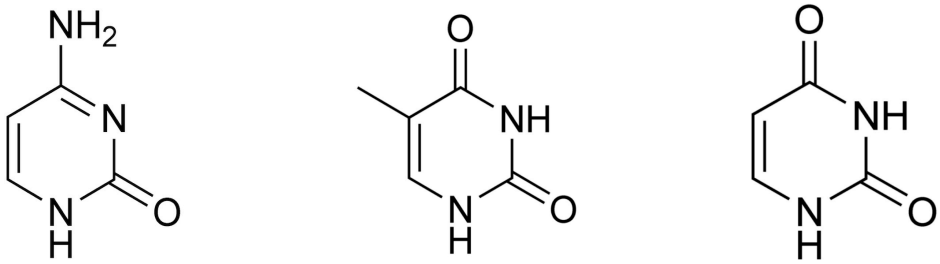
CHAPTER I

INTRODUCTION

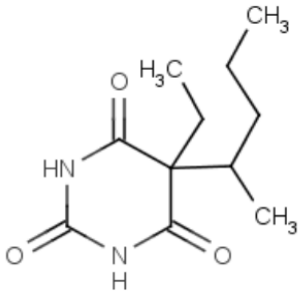
Pyrimidines are organic aromatic compounds containing two nitrogen atoms within the single ring structure. Common pyrimidine derivatives include cytosine, thymine, and uracil, from which some nucleic acids are derived.



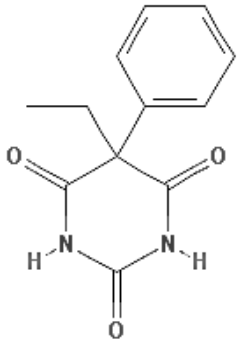
Cytosine Thymine Uracil

Figure 1: Pyrimidine derivatives that are common nitrogenous bases found in DNA and RNA.

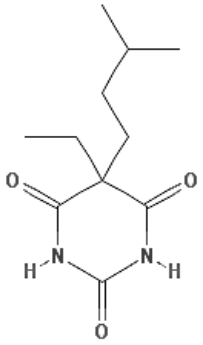
These nitrogenous bases are commonly found in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Pentobarbital, 5-ethyl-5-(1-methylbutyl)-barbituric acid, is a substituted pyrimidine derivative in a class of drugs called barbiturates (Figure 2).



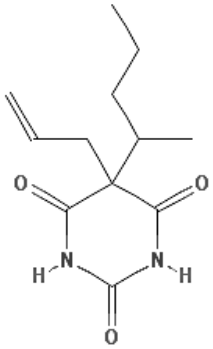
Pentobarbital



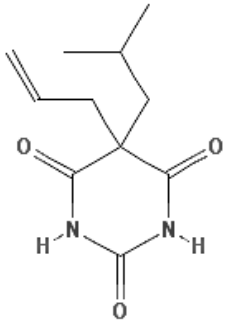
Phenobarbital



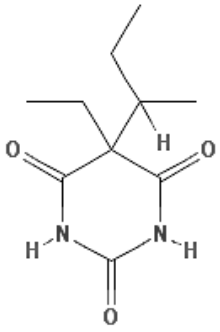
Amobarbital



Secobarbital



Butalbital



Secbutalbarbital

Figure 2: Barbiturates are a class of drugs derived from pyrimidines that affect and depress the central nervous system. Several thousand derivatives of barbiturates have been synthesized. The above images are a selection of the more common barbiturates taken from the Public Chemical Database (NCBI, 2013).

Barbiturates are perhaps more commonly known for their depressant effects on the central nervous system (CNS), but they actually have a broad range of therapeutic effects. They affect and "depress the sensory cortex, decrease motor activity, alter cerebellar function and produce drowsiness, sedation and hypnosis" (Oak Pharmaceuticals, Inc, 2012). The mechanism of action is thought to be in part due to the barbiturates effect on the neurotransmitter γ-amino-butyric acid (GABA) (American Society of Health Systems Pharmacists, 2009). This is the brain's major inhibitory neurotransmitter in the mammalian nervous system. A neurotransmitter is a chemical substance that is released from the axonal end of one nerve cell where it diffuses across a gap to the dendrite end of another nerve cell. The gap between the axonal end and the dendrite end is known as the synaptic cleft. Specific receptors or transmembrane molecules on the surface of the receiving cell bind the neurotransmitter and subsequently send a signal inside the cell to do something (Voet et al., 2008). Barbiturates alter the synaptic transmission that is mediated by the GABA receptors and in turn affect the inhibitory response of the nervous system. Although the mechanism of action is still under investigation, it appears as though barbiturates act by prolonging the duration of the channel opening of the GABA receptor (American Society of Health Systems Pharmacists, 2009).

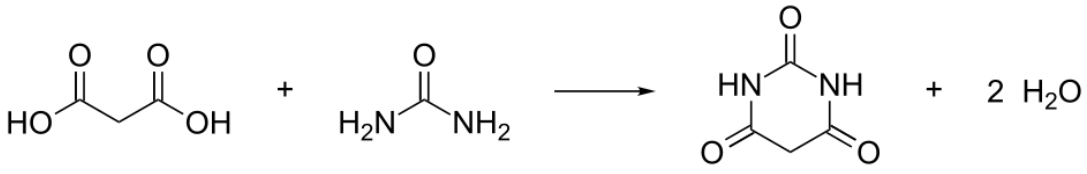
The first recognized medicinal benefit of barbiturates was the hypnotic effect. For the first time, patients who were at one time untreatable were suddenly able to receive treatment due to the repression of their emotions and inhibitions (Lopez-Munoz et al., 2005). The introduction of barbiturates brought about a revolution in the treatment of

psychological and neurological disorders. It was later discovered that barbiturates were successful in treating sleep disorders as well as being the first truly successful treatment of epileptic seizures (Lopez-Munoz et al., 2005).

Barbiturates have been extensively used throughout the United States, but due to the alarming rate of dependence and death associated with barbiturates, they are used far less than in years past. One prominent example was the death of 1950's American film star Marilyn Monroe. Her death was linked to an acute poisoning by pentobarbital. However, many derivatives of barbiturates are still frequently used today for specific therapeutic treatments, such as for the treatment of epilepsy, preoperative anxiety, insomnia, psychiatric disorders and to a lesser extent the treatment of hemolytic jaundice, post-surgical cerebral edemas or swelling, and cardiac ischemia or the decrease in blood supply to the heart due to an obstruction in the artery (Lopez-Munoz et al., 2005).

Barbiturates first became commercially available in 1904, but it was forty years prior that barbiturates were synthesized. In 1864, Adolf van Baeyer, founder of the Bayer Chemical Co. and recipient of the Nobel Prize in Chemistry in 1905, was the first to synthesize a barbiturate (Lopez-Munoz et al., 2005). French chemist Edouard Grimaux perfected the synthesis in 1879 and soon after, Conrad and Guthzeit synthesized the first clinical form of barbiturate, diethyl-barbituric acid (Lopez-Munoz et al., 2005). Twenty-three years later, German companies E. Merck and F. Bayer and Company introduced the hypnotic drug called "barbital" (Lopez-Munoz et al., 2005).

Chemically barbiturates are considered to be closed chain ureic compounds, with a malonylurea nucleus (Lopez-Munoz et al., 2005). Barbituric acid is synthesized from the combination of malonic acid and urea (Figure 3).



Malonic Acid Urea Barbituric Acid

Figure 3: Synthesis of barbituric acid from malonic acid and urea.

Interestingly, barbituric acid is pharmacologically inactive and therefore has no effect on the central nervous system. It is the substituted pyrimidine ring of barbituric acid that produces CNS effects (Oak Pharmaceuticals, Inc, 2012).

Several thousand derivatives of diethyl-barbituric acid were synthesized with far more reaching effects and more flexible durations of action. According to Taber's Cyclopedic Medical Dictionary, drug action is the function of a drug in various body systems (Taber, 1997). Duration of action refers to the length of time the drug redistributes throughout the body and affects the target system, in this case the central nervous system. While this varies from person to person, some barbiturates have longer duration of actions than others and are classified accordingly. Additionally, the sodium salts absorb more readily than do their acid counterparts regardless of the method of

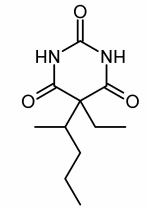
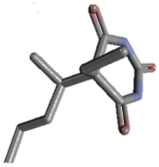
administration (National Library of Medicine, 2010). The onset of action varies according to the lipid solubility of the barbiturate. The more lipid-soluble the barbiturate the faster it distributes through the tissues, especially the brain, liver and kidneys (Oak Pharmaceuticals, Inc, 2012; American Society of Health Systems Pharmacists, 2009). Further, it is not the metabolism of barbiturates that primarily effects the duration of action but rather the redistribution of the barbiturate into the total body water and tissue binding sites including fat, that results in lower plasma concentration and concomitantly the amount in the brain (American Society of Health Systems Pharmacists, 2009). There seems to be an interest in moving away from the classification of barbiturates according to their duration of action (short, intermediate, or long) to their intended pharmacological use such as sedative hypnotic or as an anesthetic (National Library of Medicine, 2010). Phenobarbital, for example, is a barbiturate used in the treatment of seizures and has the slowest onset of action and a long-lasting effect on the central nervous system with a drug action lasting up to, on average, 79 hours (Oak Pharmaceuticals, Inc, 2012). It is the least lipid soluble and consequently the slowest to cross the blood brain barrier (American Society of Health Systems Pharmacists, 2009). It can take up to five days in adults to be completely eliminated from the human body (Kwan and Brodie, 2004).

Pentobarbital is synthesized from a condensation reaction of a substituted malonic ester (1-methyl butyl-ethyl malonic ester) and urea followed by hydrolysis to give the resulting barbital compound (Table 1). The International Union of Pure and Applied Chemistry (IUPAC) name for pentobarbital is 5-ethyl-5-(pentan-2-yl)-1,3-diazinane-

2,4,6-trione. Other names include 5-ethyl-5- (1-methylbutyl)-barbituric acid and 5-ethyl-5-(1-methylbutyl)-2,4,6,-trioxohexahydropyrimidine.

Pentobarbital is categorized as a fast-intermediate sedative-hypnotic drug. It is highly lipid soluble 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 is obtained within 15 - 60 minutes if administered orally and within a minute if administered intravenously (American Society of Health Systems Pharmacists, 2009). According to the National Library of Medicine Database, "the plasma concentrations of pentobarbital decline in a biphasic manner" (American Society of Health Systems Pharmacists, 2009). The half-life of the distribution phase or alpha phase is about one to four hours if administered orally and only 15 minutes if administered intravenously and elimination or the beta phase occurs in approximately 35-50 hours (American Society of Health Systems Pharmacists, 2009).

Table 1: Structure and Properties of Pentobarbital

	
IUPAC	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	Nebutal
Common Form	Sodium Pentobarbital
Molecular Formula	$C_{11}H_{18}N_2O_3$
Molecular Weight	226.27
pKa	7.8
Solubility in Water	679 mg/L (25 ℃)
Melting Point	129.5 ℃
Classification	Fast-Intermediate Barbiturate
Legal Status	Class II
Onset of Action	Intravenously: seconds
	Orally: 15 – 60 minutes
Duration of Action	
Alpha Phase	15 minutes if administered intravenously
	1 – 4 hours if administered orally
Beta Phase	35 – 50 hours
Dosage	Hypnotic Dosage – 100 mg
Maximum Daily Dosage	200 mg
Lethal Dosage	2 - 10 g

Barbiturate usage peaked in the 1930's and 1940's in America. In 1947, at the height of production, more than 900,000 pounds of barbiturates were produced in a single year in the United States (Lopez-Munoz et al., 2005). According to Lopez-Munoz et al., "Barbiturate use in the pre-benzodiazepine period was such that, in the USA alone, production of these drugs reached, in 1955, the quantity necessary for the treatment of 10 million people throughout an entire year" (Lopez-Munoz et al., 2005). In just a span of two decades (1941-1960), more than 15 million pounds of barbiturates and barbiturate derivatives were produced in the United States (Lopez-Munoz et al., 2005). Although the human use of barbiturates has waned, pentobarbital is still used extensively throughout the United States for medicinal purposes. It is frequently used in the medical field as a preoperative depressant and as an emergency treatment for seizures. However, its more popular use has fallen outside of the medical field and into the veterinary field.

Pentobarbital is the leading method for euthanizing animals, in particular large farm animals (Wolfgang et al., 2009). Concerns about equine and bovine sufferings from alternate euthanasia methods led to a more humane method of killing sick or injured animals: lethal injection of pentobarbital.

In the medical field, a dose of pentobarbital is one milliliter (mL) containing 50 milligrams (mg) of sodium pentobarbital, in a solution of water and alcohol for injection (Hospira, Inc, 2009). The typical dosage is dependent upon the individual and is generally 100 mg. Acute poisoning of pentobarbital occurs in general at 10 times the normal oral dosage for hypnotic use of 100 mg (Lopez-Munoz et al., 2005; American Society of Health Systems Pharmacists, 2009). That equates to only one gram of

pentobarbital. Lethal doses range from 2 grams to 10 grams depending on the individual (American Society of Health Systems Pharmacists, 2009). However, in veterinary medicine it takes approximately 30 - 40 grams of pentobarbital to put down a mature cow or horse (Wolfgang et al., 2009). This corresponds to approximately 400x the potency of a single 100 mg dose.

At first glance, it may not appear as though the euthanasia of these large animals would have any detrimental effects. It is, after all, an attempt to be more humane. The problem occurs in the disposal of the carcass. Often disposal requires large machinery to bury the animal carcass or specialty services such as crematories to haul the carcass away. Several methods are used to dispose of euthanized horses including burial, composting, rendering, cremations and landfills. Each of these methods can be costly and in certain states, some of these methods are illegal (The Humane Society of the United States, 2013). However, once an animal is euthanized, it is essential to dispose of the carcass properly. Veterinary case reports of secondary contaminations and deaths involving wild animals, birds of prey and pets scavenging these carcasses are numerous. (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, reports also exist in which animals were poisoned by meat fed to them from rendering euthanized animals. Three Sumatran tigers in Heidelberg Zoo in Germany were poisoned by contaminated meat fed

them from a horse euthanized with pentobarbital, and in another instance a lioness was poisoned in a similar fashion (Jurczynski and Zittlau, 2007; Verster et al., 1990).

Prior work has made significant gains in revealing the dangers associated with using pentobarbital as the lethal cocktail for livestock. Most obvious were concerns about pentobarbital making its way into the food supply chain as a result of rendering these euthanized animals. The threat of contamination is serious due to the stable nature of pentobarbital. It survives the rendering process without undergoing degradation and partitions equally into fat and protein (Myers, 2009; O'Connor et al., 1985). An FDA research team at the Center for Veterinary Medicine studied the safety of feed products for animals. The data supported findings of pentobarbital in dog food samples as confirmed by gas chromatography / mass spectrometry (GC/MS) as well as liquid chromatography / mass spectrometry (LC/MS) (Adam and Reeves, 1998; Heller, 2000). Pentobarbital was detected at confirmable amounts in commercial feed likely resulting from the euthanasia of large animals and the subsequent disposal of the carcass by rendering (Myers, 2009). Due to the animal's large size and the amount of pentobarbital necessary for euthanasia, the carcass would result in a significant amount of pentobarbital rendered into meat and bone meal. Factors affecting the amount of pentobarbital in commercially prepared pet foods would depend on several factors including the amount of pentobarbital used to euthanize the animal, the mixing of raw materials at the facility rendering the meat, the distribution of pentobarbital-containing carcasses among the "cookers" or vats, and the proportion of meat and bone meal as a source of protein to that of other ingredients used in the feed (O'Connor et al., 1985). In 2002, the European

Commission placed restrictions and regulations on the use of psychotropic drugs in animal feed (Wang et al., 2010; Commission Decision 2002/657/EC, 2002; Zhao et al., 2006) In 2003, the FDA followed a similar course of action establishing the Animal Feed Safety Action to protect animal health and human health and then again in 2009 updating the restrictions placed on rendering plants prohibiting the use of rendered feed ingredients that contain harmful chemical substances, toxins or microorganisms (Wang et al., 2010; U.S. Food and Drug Administration, 2009; US Food and Drug Administration, 2010). However, the FDA does not prohibit the rendering of animals that have died from causes "otherwise than by slaughter," only the rendering of animals that pose a risk of disease transmission to the animals that are fed the rendered product and/or the humans that consume their edible products (US Food and Drug Administration, 2010). Further, the practice of using meat and bone meal is common in many countries and reports of pentobarbital in animal feed are numerous. In Canada alone, more than 89,000 horses were processed in 2010 for meat (Horse Welfare Alliance of Canada, 2010). According to Wang and co-workers "The consumer food safety concern about barbital survival in animal feed and whether the residues could occur in animal-derived tissues is still serious. The need to monitor their presence in biological tissues, environmental samples and food stuffs is obvious" (Wang et al., 2010).

Along with concerns about the food supply, there is concern over the effect of euthanized carcasses on the environment. According to Cornell Waste Management Resources, more than 900,000 horses must be disposed of annually in the U.S. (Bonhotal et al., 2012). In addition to the increasing number of horses, cows, donkeys and other

large animals euthanized each year, more than three million stray cats and dogs from animal shelters are also euthanized. Couple this with the euthanasia of sick or aged companion pets and the veterinary usage of pentobarbital alone is staggering. Due to FDA regulations, rendering plants are limited in what they will accept and this has created new problems in the disposal of the pentobarbital-containing carcasses. One growing and cost effective method is disposal by burial or composting. However, in some states, there are restrictions as to the proximity of the burial site to sources of water and it is illegal to bury a euthanized animal in some areas (Nebraska Horse Council, 2009). Recent studies have questioned the environmental effect of burying carcasses euthanized with pentobarbital. It was reported in one study examining the pentobarbital residues in compost piles containing euthanized carcasses, that compost samples tested positive for pentobarbital residues within days of burying the euthanized carcass and over time additional samples showed increases in concentration (Cottle et al., 2009). In another study, researchers examined bovine liver tissue spiked with 125 mg of pentobarbital and buried in static and active compost piles and tested over a period of 80 days. Initial results of the static compost samples indicated an 80% recovery of pentobarbital in the tissue (Wolfgang et al., 2009). The research is limited, but initial results suggest burial leads to the leaching of pentobarbital from the animal tissue into the surrounding soil and water supply. Another concern is how long it takes pentobarbital to break down, if at all. In a case of secondary poisoning from pentobarbital, two dogs happened upon an unburied horse carcass in a ravine. The horse had been euthanized with pentobarbital and dumped in the ravine more than two years earlier (Kaiser et al., 2010). Although the

horse had been scavenged significantly, one dog ingested a lethal dose, which is reportedly 85 mg/kg for dogs (Kaiser et al., 2010). It may be possible that the leaching of pentobarbital into ground water and soil may correlate to burial and decay of the carcass, but it is apparent that pentobarbital resists degradation. Studies of this nature are also limited, likely overshadowed by the influx of human pharmaceuticals into the environment (Wang et al., 2010). It is important to consider the diverse entry of pentobarbital into the environment.

Human and veterinary pharmaceuticals are entering the environment at an alarming rate. They are released mainly from manufacturing processes, disposal of expired or unused products and excreted metabolites (Diaz-Cruz et al., 2003). The occurrence of drugs in the environment are in part due to several factors including the amount manufactured, the dosage administered, the excretion efficiency of the parent compound and its metabolites, the sorption in soils and the degradation of the pharmaceutical (Diaz-Cruz et al., 2003). However, veterinary pharmaceuticals have an increased potential for reaching the soil environment based on their usage (Tolls, 2001). Further, the persistence of pharmaceuticals in the environment can contribute to increasing concentrations or interactions with other pharmaceuticals. The persistence of a pharmaceutical in soil or sediment depends primarily on its photostability, its binding and sorption capabilities and its decay rate (Diaz-Cruz et al., 2003). Photolysis is the decomposition or break down of a molecule as a result of absorbing light energy.

Pentobarbital is not susceptible to photolysis due to its lack of chromophores or its inability to absorb light in the visible spectrum (Lyman, 1990). It is also thought to

have a high degree of mobility in the soil based on an estimated value of 28 of the soil organic carbon-water partitioning coefficient or Koc (Hansch et al., 1995). Koc is the affinity of organic molecules such as pentobarbital to sorb to the soil particles or sediment. Specifically, it is the fraction of the mass of the contaminant, such as pentobarbital, that is absorbed in the soil versus the mass of organic carbon in the soil per the concentration of contaminant remaining in solution i.e. water after equilibrium. For this reason, Koc values are used in predicting the mobility of organic soil contaminants. If Koc values are high, then a high degree of contaminant is absorbed by the soil and the contaminant is considered less soluble in water and therefore less mobile while lower Koc values correlate to a smaller absorption into the soil and a higher degree of solubility in water and thus more mobility (Kerle et al., 2007). It has also been documented that anions generally do not absorb as well to soils containing organic carbon and clay as they do to more neutral soils such as sand. For this reason, the partition coefficients for a compound can vary significantly depending on the type of soil (Tolls, 2001). Other factors also affect the sorption of pharmaceuticals to soils. According to Tolls, "A number of hydrophobicity-independent mechanisms such as cation exchange, cation bridging at clay surfaces, surface complexation, and hydrogen bonding appear to be involved" and that Koc values do not account for these processes (Tolls, 2001). Diaz-Cruz et al. reinforces Tolls statement in confirming that "sorption to organic matter, surface adsorption to mineral constituents, ion exchange, complex formations with metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+}, or Al^{3+} and hydrogen bonding" affect drug sorption (Diaz-Cruz et al., 2003). The reported pKa of pentobarbital is 7.8 suggesting that it exists, to a

limited extent, in the anion form in the environment and is therefore less likely to be absorbed by soil (Wollweber, 2008; Doucette, 2000). Compounds that sorb weakly to soil or sediment are more susceptible to uptake by ground water and less available for microbial degradation and plant uptake (Kerle et al., 2007). Although research is scarce, one study has shown that pentobarbital does leach into the ground water. In Jacksonville, Florida, ground water was taken from a well near a landfill, which received wastes in the late 1960s. The ground water tested positive for pentobarbital more than 15 years following the landfill's usage. The well water was tested again, after a period of 22 years following the time it received wastes and pentobarbital persisted at a concentration of 1 μg/L, which corresponds to 1 ppb (Eckel et al., 1993).

Biodegradation data is not available for pentobarbital according to Hazardous Substances Data Bank (HSDB). In general, research is very limited on the microbial degradation of barbiturates. In 1951 and 1952, two studies, one by Wang and Lampen from Western Reserve University, and the other by Hayaishi and Kornberg of the National Institutes of Health respectively, were conducted to determine the metabolic transformation of pyrimidines by soil bacterium. They discovered a soil bacterium, later named *Rhodococcus erythropolis* JCM 3132, that successfully metabolized pyrimidines (Soong et al., 2002). It was noted that JCM 3132 could oxidize all 5-substituted compounds of uracil or cytosine and that the oxidation of the pyrimidine ring likely involved the carbon 6 position (Wang and Lampen, 1951). Their work only identified the metabolic pathway to a very limited extent. It was Hayaishi and Kornberg's research that revealed that bacterial enzymes were involved in the metabolism of pyrimidines. An

enzyme, “barbiturase” found in the bacterium *Rhodococcus erythropolis* is the key to metabolizing barbituric acid to urea and malonic acid (Hayaishi and Kornberg, 1952). The precise way in which barbituric acid was metabolized was still undetermined (Hayaishi and Kornberg, 1952). By definition, an enzyme is a biological catalyst (Voet et al., 2008). “Enzymes accelerate biochemical reactions by physically interacting with the reactants and products to provide a more favorable pathway for the transformation of one to the other” (Voet et al., 2008). Enzymes are protein-based molecules that specifically bind to a complimentary substrate that is geometrically and electronically favorable.

Pyrimidines are metabolized one of two ways: either the reductive or oxidative pathway (Figure 4). In humans, pyrimidines and pyrimidine derivatives, like pentobarbital, are metabolized in the liver via the reductive pathway to yield the inactive metabolites pentobarbital carboxylic acid, parahydroxyphenyl derivative, and 5-ethyl-5(3’-hydroxy-1’-methylbutyl) barbituric acid, or commonly called hydroxypentobarbital which results from the oxidation of the 1-methylbutyl substituent (Figure 4A) (American Society of Health Systems Pharmacists, 2009). According the AHFS Drug Information, 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).

Some microorganisms alternatively metabolize pentobarbital via the oxidative pathway. In 2001, a study conducted on the pyrimidine oxidation degradation in JCM 3132 by Chee-Leong Soong et al. in Japan, made significant gains in elucidating the oxidative metabolic pathway of pyrimidines (Figure 4B).

In the oxidative pathway, barbituric acid is converted via the enzyme barbiturase to ureidomalonic acid and then finally to malonate and urea by another enzyme called ureidomalonase discovered by Soong et al. in 2001 (Soong et al., 2002). In their work, they were able to purify and characterize the enzyme barbiturase and then sequence it. No prior homology was found relating to barbiturase and in as much they suggested that barbiturase belonged to a novel amidohydrolase protein superfamily.

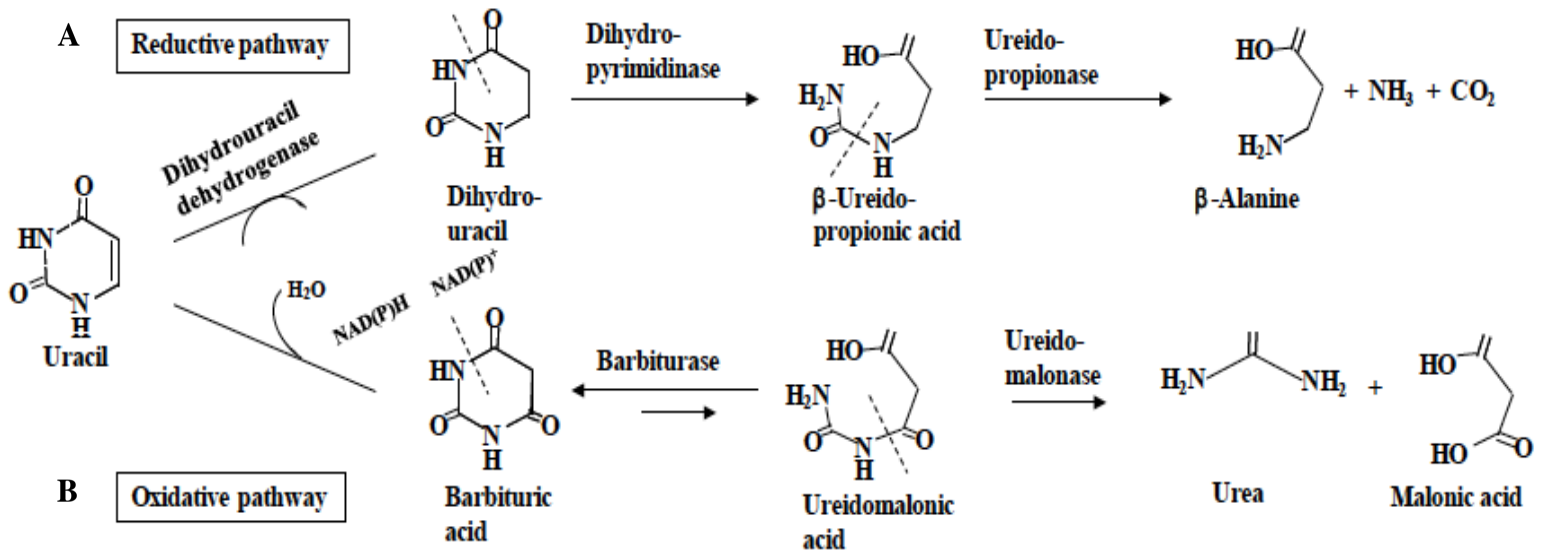


Figure 4A: Reductive pathway in microbial metabolism of pyrimidine base as elucidated by Chee-Leong Soong et al. in 2001.

Figure 4B: Oxidative pathways in microbial metabolism of pyrimidine base as elucidated by Chee-Leong Soong et al. in 2001.

It is surprising that in over sixty years since its discovery very little work has been done to discover the detailed mechanism of this enzyme. With approximately 100 million to 1 billion bacterial cells in a teaspoon of soil, it is even more surprising that no other bacterium have been identified as capable of degrading barbiturates such as pentobarbital (Ingham, 2013).

Literature detailing a method of detection for barbiturates, specifically pentobarbital, utilizing liquid chromatography / mass spectrometry (LC/MS) is scarce. LC/MS offers a faster and more flexible analysis without the need for derivatization required by GC/MS (Heller, 2000). Derivatization is the process of transforming a nonvolatile analyte through a chemical reaction into a volatile or more reactive form so that is can undergo analysis such as gas chromatography.

Methods for detecting chemical substances such as pharmaceuticals are developed for multiple reasons, some of which are apparent. Methods may be developed to study the metabolic pathway of a drug and the metabolites resulting from the metabolism of these drugs. Methods are also developed out of a need to study the efficacy of the drug and the effect after extended usage. However, what may not be apparent is each method is generally unique and specific to the need. Most method developments stem from the need to study the effects drugs have on people, and this typically involves a method for detecting the drug in a biological matrix such as blood, plasma or urine.

Methods for detecting barbiturates in plasma concentrations exist with respect to analyzing metabolites and pharmacologically active substances. Pentobarbital has been analyzed to this extent, as well as examining the amount of pentobarbital passed on to an

offspring in the breast milk of a lactating mother. 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 and the amount of pentobarbital in the breast milk was found to be 0.17 μg/mL after a dosage 19 hours earlier (Briggs et al., 1994).

Another method was developed after an increase in drug-facilitated sexual assaults (Frison et al., 2003). Thiopental, a barbituric acid derivative, has a quick onset of action and together with its sedative effect, short duration of action and ease of availability make it a convenient choice for perpetrators. In a case detailing the assault of a 61-year-old woman in a healthcare facility, analysis of her hair by an independent laboratory revealed traces of thiopental (Frison et al., 2003). Subsequently, a method for detecting thiopental and its metabolite pentobarbital in hair was developed by Frison et al. of University Hospital of Padova, Italy.

Other methods developed stem from occurrences in food, water supply, environment, or combinatory effects with other chemicals. Common to these methods of detection are generally techniques including high performance liquid chromatography (HPLC) or gas chromatography / mass spectrometry (GCMS). In the case of the 61-year-old woman assaulted in a medical facility, the method utilized was solid phase micro extraction (SPME) and gas chromatography / mass spectrometry/mass spectrometry (GC-MS-MS).

Chromatography is the process of separating components of a mixture. It has become one of the primary methods for identification of compounds in the gaseous or liquid state. The fundamental principle of chromatography is based on the equilibrium

between the stationary phase and the mobile phase. The stationary phase generally consists of an inert material with customized properties coated on the inside of a hollow stainless steel tube known as a column. The mobile phase is a liquid or gas carrier in which the sample or mixture is dissolved and then carried through the column under pressure. The affinity of the individual components in the mixture to the solid phase effects their migration through the column and allows for a separation of the components within the mixture or sample (Rouessac and Rouessac, 2007). The time in which the analyte or chemical of interest elutes through the column is referred to as its retention time. The identity of the chemical of interest is verified by the comparison of its retention time to the retention time of reference standards. This is generally the first method of identification. Further and more definitive identification is made through the analysis by the mass spectrometer.

A mass spectrometer is an instrument often used in tandem, although not always, with liquid or gas chromatography to characterize matter based on the mass to charge ratio of the individual species present in a sample (Rouessac and Rouessac, 2007). Essentially, a minute quantity of sample is transformed to the gas phase and then ionized to a charged species where it is then submitted to an electric or magnetic force that determines the mass to charge ratio of the ions present. In gas chromatography, the sample is already in the gaseous state. However, in liquid chromatography, the sample is dissolved into a liquid mobile phase or solvent. It is more difficult to interface liquid chromatography to mass spectrometry due to volume of solvent that must be removed before presenting the analyte to the source, but one clear advantage of using liquid

chromatography is that the sample or analyte of interest need not be volatile only thermally labile (Christian, 2004). Several methods of ionization exist. One of the most popular methods for ionizing large, polar, basic or charged molecules is an electrospray ionization source (Christian, 2004). It is considered a soft ionization technique in which it produces charged ions without a lot of fragmentation of the parent compound or analyte (Rouessac and Rouessac, 2007). This results in a small distribution of ions and therefore identification and quantification of the parent compound can be more readily determined. In a tandem mass spectrometer analysis, often a soft ionization technique is used in the first mass spectrometer to obtain the parent or molecular ion peak and the second mass spectrometer utilizes a hard ionization technique. Hard ionization produces a lot of fragmentation that is reproducible and useful for certain identification.

The FDA has been instrumental in validating the use of LC/MS for detection of contaminants, specifically in animal feed. However, there remains a need for detecting barbiturates in environmental samples. Further and just as importantly, there is a need to quantify the amount of barbiturates and their persistence in the environment especially in high occurrence areas such as agricultural areas. Although there is a growing amount of data becoming available, most literature dealing with pharmaceuticals in soils, according to Diaz-Cruz, deals with solid environmental samples, such as soils and sludge, that are contaminated as a result of antibiotics commonly used in fish farming (Diaz-Cruz et al., 2003). There is an obvious need for more research in pharmaceuticals and their emergence in the environment.

CHAPTER II

MATERIALS AND PREPARATION

2.1 Materials and Reagents

Pentobarbital sodium salt, ($C_{11}H_{17}N_2NaO_3$) (99% pure) was purchased from Sigma Aldrich Co. Pentobarbital-d_5, 5-(pentadeuteroethyl)-5-(1-methylbutyl)-2,4,6(1H,3H,5H)-pyrimidinetrione ($C_{11}H_{13}D_5N_2O_3$) (99.3% chromatographic purity) was purchased from Cerilliant Corporation. Secobarbital sodium salt, ($C_{12}H_{17}N_2NaO_3$) (≥98% pure) and sodium barbiturate, ($C_4H_3N_2NaO_3$) (≥97% pure) were purchased from Sigma Aldrich Co. Strata-X solid phase extraction units and Phenomenex ODS C18 (150 x 4.6 mm) 5 μm HPLC columns were purchased from Phenomenex Inc. Millex – GV PVDF 0.22 μm syringe driven filters were obtained from Millipore Corporation. All other chemicals were reagent grade and water was 18 MΩ.

The source of potting soil was Expert Gardener All Purpose Potting Soil Mix purchased at Lowe's Home Improvement Center. The sand, horse stall sweepings, and loam were obtained from the Department of Biology at Middle Tennessee State University. The topsoil samples A (0-10 cm) and B (11-20 cm) were obtained from the Department of Agriculture and Environmental Science at Tennessee State University.

The Department of Biology at Middle Tennessee State University provided the soil bacterium utilized in this study. All bacteria including, HSC-A, HSC-D, W2B, and alpha small, were gram negative bacilli and were isolated from soil samples taken from a horse burial mound site in Tennessee. The designation HSC- refers to the Horse Science

Center at Middle Tennessee State University. Alpha small and W2B were generic names given for identification purposes only.

2.2 Reference Materials and Working Standards

In order to quantify pentobarbital concentrations in various soil compositions, reference materials were employed to establish reliable analytical methods. A stock solution was prepared from pentobarbital sodium salt and methanol. Ten (10) mg of pentobarbital sodium salt was dissolved in 1 mL of methanol and stored at 4° C. A working standard of 1 mg/mL was prepared by diluting 0.1 mL of the stock solution with 0.9 mL of methanol. Daily standards were prepared from a serial dilution of the working standard. Fresh standards were made monthly from the stock solution to ensure integrity of the standards. Further, a new stock solution of pentobarbital sodium salt in methanol was prepared biannually. Comparative analysis of the standards was conducted periodically to assess the concentration and variance in concentration from one set of standards to another.

2.3 Internal Standards

A certified internal standard was also utilized in this research. In the method development and applications of the method, a deuterated isotope of pentobarbital was utilized. A 1.0 mL/mg certified deuterated isotope standard of pentobarbital-d_5 in methanol was obtained from Cerilliant Corporation. A serial dilution of the certified deuterated isotope standard was prepared with reagent grade methanol to create

additional deuterated standards for calibration and for use as an internal standard in this research.

2.4 Construction of Calibration Curves

A serial dilution of the 10 mg/mL pentobarbital stock solution was created to establish the limits of detection and quantification for the LC/MS. Concentrations ranging from 0.0001 mg/mL to 1 mg/mL were analyzed via the LC/MS and area of the peak corresponding to pentobarbital was graphed. The coefficient of determination value was utilized to determine how well the observed values would predict unknown values.

An external calibration curve of standard reference materials was utilized for method development. An internal calibration curve was also employed for use with method development and analysis of decay of pentobarbital by the soil bacterium. The internal calibration curve was developed by taking the ratio of the area of pentobarbital and the area of pentobarbital-d_5 versus the concentration of the ratio of pentobarbital and the concentration of pentobarbital-d_5. See Chapter 4.4.

2.5 Instrumentation

The liquid chromatography mass spectrometer utilized in this research was an Agilent 1100 LC/MS System. An extensive period of trials were conducted to analyze the effects minor adjustments to the various parameters, including flow rate, gradient or isocratic elution, fragmentation voltage, ratio of mobile phase, and injection volume had on the noise levels, number of peaks, peak area of pentobarbital, Gaussian distribution of

the pentobarbital peak and the ions present in the spectrograph of the pentobarbital peak. After optimal conditions were confirmed utilizing standards of pentobarbital sodium salt, LC/MS method parameters were established and detailed below.

A 60/40 ratio of acetonitrile and 18Ω water was used for the mobile phase. The injection volume was 20 μL. The pump was programmed for isocratic elution with >10 minutes between injections to allow the column to equilibrate. The flow rate was 0.6 mL/min for 0 - 6 minutes. It then increases to 1.0 mL/min from minutes 6 - 7 and runs at 1.0 mL/min for minutes 7 - 8 minutes and then returns to 0.6 mL/min over minutes 8 - 9 and then continues at 0.6 mL/min for the remaining time. The retention time for pentobarbital was 3.4 minutes for both standards and samples. The ionization mode was electrospray-atmosphere pressure ionization (ES-API) and the fragmentation voltage was 90. Under negative polarity the ion spectra for pentobarbital [M-H]- is 225m/z.

A Hitachi U2900 UV spectrophotometer, at a wavelength of 256 nm, was utilized for kinetic analysis of the alpha small bacteria degradation of pentobarbital.

2.6 Soil Sample Preparations and Handling

Heterogeneous soil mixtures 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. Topsoil samples, 0-10 cm and 11-20 cm, horse stall sweepings and loam were ground with mortar and pestle to produce a more uniform particle size. This step was not necessary for sand and potting soil samples, as they were consistently uniform in particle

size. In order to spike the soil samples with the desired concentration, a working solution of pentobarbital was prepared from the 10 mg/mL stock solution of pentobarbital. For example, if soil samples were spiked with 0.1 mg of pentobarbital each, a master solution was prepared by taking the appropriate amount of the stock solution and diluting with methanol to create the necessary volume of working solution with a concentration of 0.1 mg/mL. From this working solution, 1 mL was used to spike each sample of soil. Five-gram samples of potting soil, topsoil A (0-10 cm depth), topsoil B (11-20 cm depth), sand, horse stall sweepings and loam were spiked with the desired concentration as necessary.

Long Term Soil Preparation

Three master samples containing 35 grams of potting soil, sand and topsoil (11-20 cm) respectively were each spiked with 0.07 mg of pentobarbital and mixed thoroughly. The master samples were each divided into seven 5 g samples and stored in a 50 mL centrifuge tube and incubated at 37 ºC over a 17-week period of sampling.

Dispersion Soil Preparation

A 5 gram sample of potting soil was placed in a 10 mL syringe with filter. The top surface of the soil was then exposed to 1 mg of pentobarbital sodium salt. A 10 mL volume of distilled water was then pulled through the syringe under vacuum. This was repeated two more times for a total of three column volumes of water. Each of the three filtrates were collected separately and stored for analysis. The soil within the syringe was

then separated into three layers, the top layer consisting of the first cm, the second layer the next three cm, and the third layer containing the last 3.75 cm.

2.7 Bacteria Preparation

The bacteria utilized in this study were taken from healthy colonies grown on minimal agar supplemented with pentobarbital. A 10 mL aliquot of a sterile solution of minimal broth (l L) without dextrose containing 1 mg of zinc chloride and the desired concentration of pentobarbital sodium salt was inoculated with bacteria from the selected colonies. Bacteria samples were then prepared with 24 mL of the minimal solution and 1 mL of the bacteria in minimal mixture. The 25 mL samples were placed in an incubator at 37 ºC and 200 rpm for a period of days. The optical density, at 600 nm, of 1 mL extracts were analyzed daily for bacteria growth. After five days, bacteria samples were removed from the incubator and centrifuged at 10,000 RCF (relative centrifugal force) for 10 minutes. The supernatant was then stored for analysis of pentobarbital concentration using the method developed. The bacteria cells were suspended in 0.5 mL of sterile 20 mM potassium phosphate buffer (pH 7.0) and sterile glycerol and stored at -70 ºC.

Analysis of Barbiturase Activity (Kinetic Activity)

Alpha small cells were suspended in 10 mL of 20 mM potassium phosphate buffer solution (pH 7.0). Cells were disrupted in 10 second intervals with a bead beater, 0.50 mm diameter glass beads, at 5 ºC for 2.5 minutes. The solution was then centrifuged

at 10,000 RCF for 25 minutes at 4 ºC to eliminate the cellular debris. The supernatant containing the enzyme was collected and a 10 μL alliquot was injected into a quartz cuvette containing a 900 μL volume of 0.1 mM solution of pentobarbital and 20 mM potassium phosphate buffer solution (pH 8.0). Kinetic activity was measured on a Hitachi U2900 UV spectrophotometer by monitoring the decrease in absorbance at 256 nm for 20 minutes.

CHAPTER III

METHOD DEVELOPMENT

3.1 Extraction of Pentobarbital from Soil

Laboratory grade methanol (25 mL) was added to the prepared soil samples and mixed thoroughly by automated shaking overnight. The sample was allowed to settle and the liquid was decanted into a 50 mL centrifuge tube. Another 10 mL of methanol was added to the soil sample, and again mixed thoroughly by shaking for one hour and allowed to settle. The liquid was removed and combined with the original methanol. The methanol solution was centrifuged at 1900 rpm for 15 minutes to remove particulate matter. The supernatant was transferred to a fresh centrifuge tube. This process was repeated once more and the combined supernatants were evaporated to near dryness on a rotary evaporator at 37 ºC. An additional 5 mL of water was then used to re-dissolve the extract. It was then filtered through a Millex – GV PVDF 0.22 µm syringe driven filter. The filtrate was then ready for solid phase extraction.

3.2 Solid Phase Extraction (SPE) Method

Strata-X 33µm polymeric reverse phase extraction cartridges were activated and conditioned with 2 mL 5% methanol, then washed with 2 mL 0.1M sodium acetate buffer (pH 7.0). The pentobarbital sample was then passed through the cartridge at a rate of 5 - 8 drops per 10 seconds. The cartridge was then washed with 1 mL of 0.1 M sodium acetate buffer and allowed to dry under vacuum for a minimum of 5 minutes. The pentobarbital

was eluted from the Strata-X SPE into a fresh flask using 1.1 mL of 50:50 20% methanol/acetonitrile. The solution was then transferred to an HPLC vial for LC/MS analysis.

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

Samples were chromatographed on a Phenomenex ODS C18 150 x 4.6 mm 5 μm column on an Agilent 1100 LC/MS. The method parameters are detailed in Appendix A. The pump was programmed for isocratic elution with >10 minutes between injections to allow the column to equilibrate. The retention time for pentobarbital was 3.4 minutes for both standards and samples. The ion spectra for pentobarbital [M-H]- is 225m/z.

3.4 Detection, Long Term Analysis, Dispersion and Biodegradation Methods

Detection of Pentobarbital in Soil

Several types of soil were analyzed for pentobarbital utilizing the method detailed above. Potting soil, sand, topsoil (0 – 10 cm), topsoil (11 – 20 cm), loam and horse stall sweepings were all processed without modifications to the method.

Long Term Analysis of Pentobarbital in Soil

Soil samples in the long term analysis were incubated in individual vials over a period of 17 weeks and were processed according to the method without variation.

Dispersion of Pentobarbital in Soil

Soil samples from the dispersion analysis were processed according to the method with one exception. Due to the small volume of soil contained within each layer, generally no more than 2 grams, only a fraction of methanol was used to extract the pentobarbital from the each layer.

Dispersion of Pentobarbital in Water

A 1 mL aliquot of the filtrate stored from the dispersion analysis was analyzed with a slight modification to the method. Due to the aqueous nature of the sample, the extraction procedure was omitted. The sample was processed directly by solid phase extraction of pentobarbital and determined by LC/MS.

Biodegradation of Pentobarbital via Bacterium

A 1 mL aliquot of the supernatant from the bacteria samples was diluted to 5 mL with 18Ω water and filtered through a Millex – GV PVDF 0.22 μm syringe driven filter. Samples were then processed with the solid phase extraction and LC/MS procedures detailed in the method.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Purpose

Decades of usage have certainly created the need for a more thorough understanding of the amount of pentobarbital in our environment, the possible health risks associated with this contamination, the potential for additive effects of pentobarbital alongside other pharmaceuticals as they find their way into the soil and water supply and, if possible, a method of remediating soil contaminated with pentobarbital. According to Wang, the additive effect of mixing these pharmaceuticals with other chemical contaminants remains unknown. (Wang et al., 2010)

The primary purpose of this research was to develop a method utilizing liquid chromatography / mass spectrometry to detect pentobarbital in a variety of environmental samples, including but not limited to potting soil, top soil, sand, loam, stall sweepings and water. Secondary studies include the application of this method in the analysis of the decay of pentobarbital, the effects different soils have on recovery and decay of pentobarbital, and the analysis of a soil bacterium potentially capable of breaking down pentobarbital. The determination of pentobarbital in soil and other matrices is the first step to quantifying the amount of pentobarbital in the environment and then examining its degradation.

This method of extracting pentobarbital out of complex matrices, specifically soil, is effective for minute amounts and adaptable to other barbiturates and applications. The

method was analyzed with the barbiturate derivatives pentobarbital, secobarbital and barbituric acid in addition to analysis with complex aqueous mixtures.

4.2 LC/MS Sensitivity, Optimization, Limits of Detection

In the development of this method, a thorough analysis of varied LC/MS operating conditions was conducted using pentobarbital standards. An ODS C-18 column was employed at a column temperature of 40 ºC. The mobile phase consisted of a 60/40 ratio of acetonitrile and water respectively. An isocratic elution with a flow rate of 0.4 mL/min was successful in separating the components (Figure 5). Small adjustments (0.4 to 0.6 mL/min) could be made to improve separation and not compromise the Gaussian peak of the chromatograph. The following table details the parameters for the LC/MS that produced the most reliable and reproducible data (Table 2).

The most significant factor was utilizing negative polarity with an electrospray ionization mode. No peaks were present when LC/MS conditions were set to positive polarity.

Table 2: LC/MS parameters established for the detection of pentobarbital according the method.

LC/MS METHOD PARAMETERS	
Column	ODS C-18
Elution	Isocratic
Mobile Phase	Acetonitrile/Water
M.P. Ratio	60/40
Flow Rate	0.4 mL/min
Ionization Mode	ES-API
Polarity	Negative
Column Temperature	40°C
Fragmentation Voltage	90
Retention Time	3.4 min
$[M-H]^-$	225 m/z

In the following figure, a total ion chromatograph of a topsoil sample spiked with pentobarbital illustrates how separation of the organic components remaining in the sample was achieved by applying the detection method and optimizing the operating conditions of the LC/MS (Figure 5). Pentobarbital has a retention time of approximately 3.4 minutes, correlating to the peak at 3.46 minutes in the chromatograph.

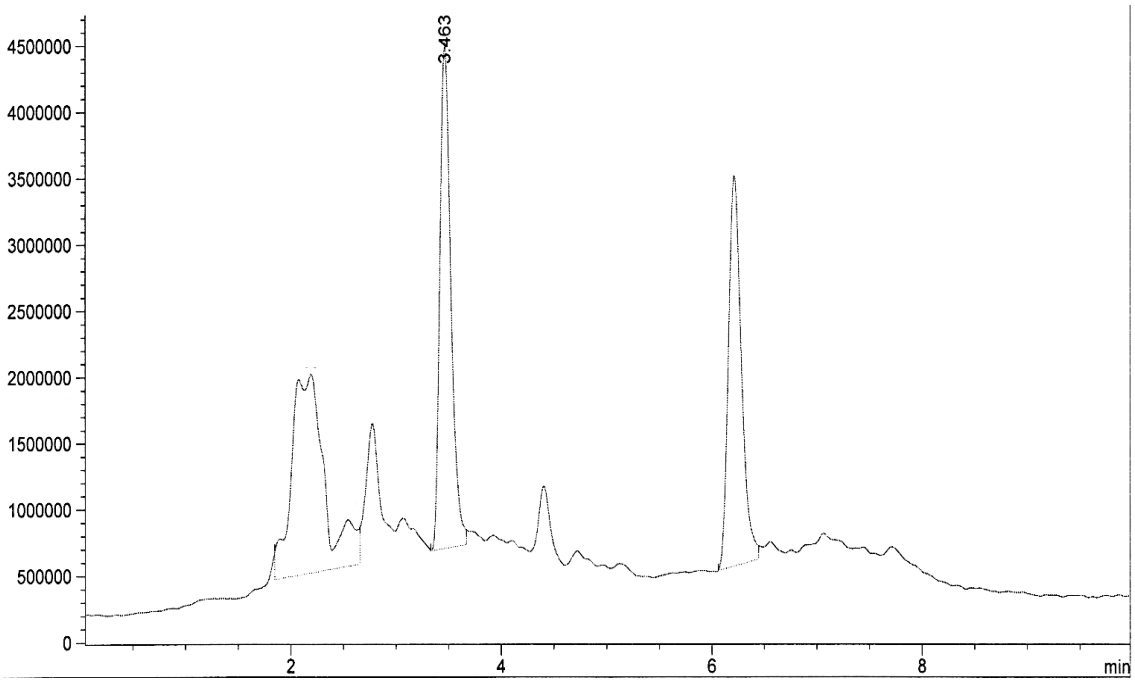


Figure 5: Total Ion Chromatograph (TIC) of a topsoil sample analyzed via LC/MS after application of the method. Pentobarbital had a retention time of approximately 3.4 minutes for standards, soil samples and bacteria samples. Good separation of components was achieved with a flow rate of 0.4 mL/min.

The following figure is the mass spectrum of the ions present in the peak (Figure 6). The detection method was effective at isolating pentobarbital as evidenced by the lack of competing ions at 3.4 min. The mass to charge ratio (m/z) of pentobarbital $[M-H]^-$ in negative mode is 225. It is the strongest peak in the spectrum. The parent compound [M] has a m/z of 226 and also present in the spectrum.

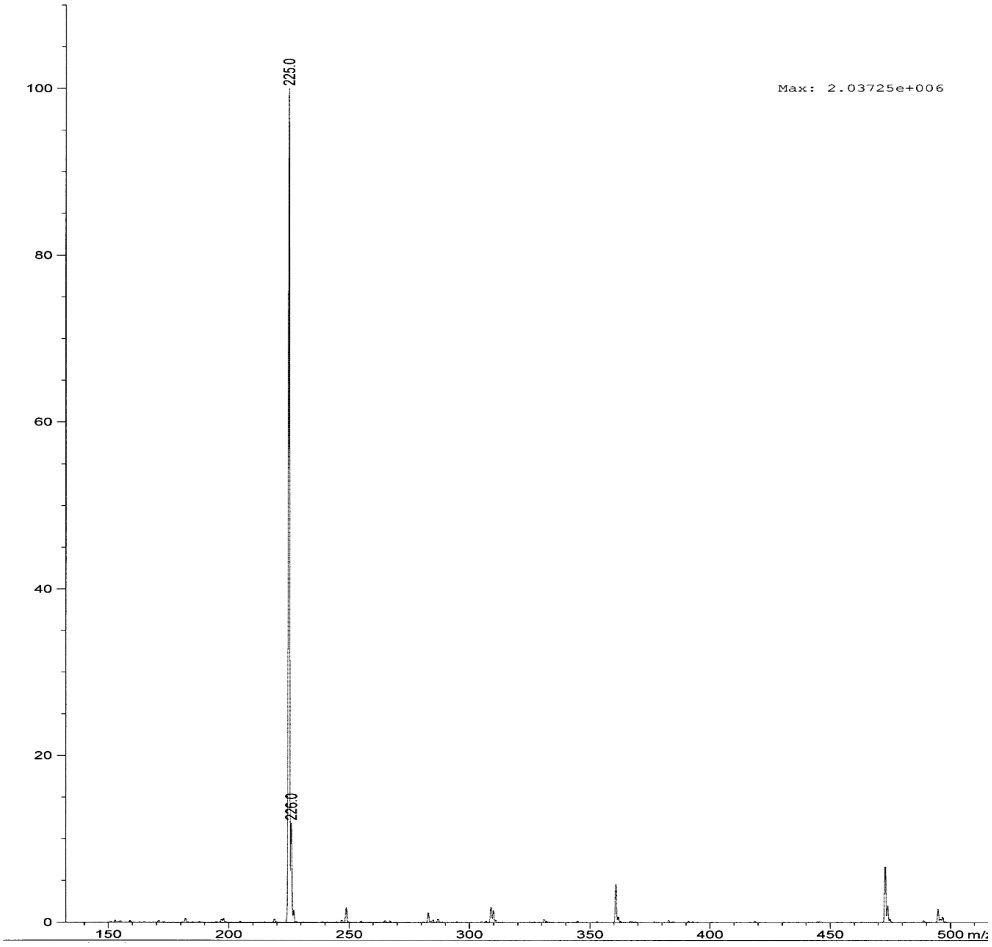


Figure 6: Ion spectra of a topsoil sample analyzed via LC/MS after application of the method. Pentobarbital has a mass to charge ratio (m/z) of 225 in negative polarity mode.

In order to establish the limits of detection for the method, a range of detection for the mass spectrometer was first established. Concentrations of 10 mg/mL (10,000 ppm wt/v) to 0.10 μg/mL (0.1 ppm) were analyzed to establish limits of detection (LOD) and a range of linearity.

The Agilent 1100 LC/MS was capable of detecting as little as 0.0001 mg/mL of pentobarbital in methanol. However, limits of quantification, or the point in which the peak area was at least 7x greater than the noise level, occurred at a concentration of 0.0002 mg/mL of pentobarbital in methanol. The upper limit for quantifying pentobarbital in methanol was 0.10 mg/mL, at which point the linearity began to degrade. It was determined that LC/MS was suitable for the analysis given the range of detection.

In the following figure (Figure 7), a chromatograph of the pentobarbital in methanol standard illustrates the peak intensity at the lower limit of detection as compared to the noise or baseline level.

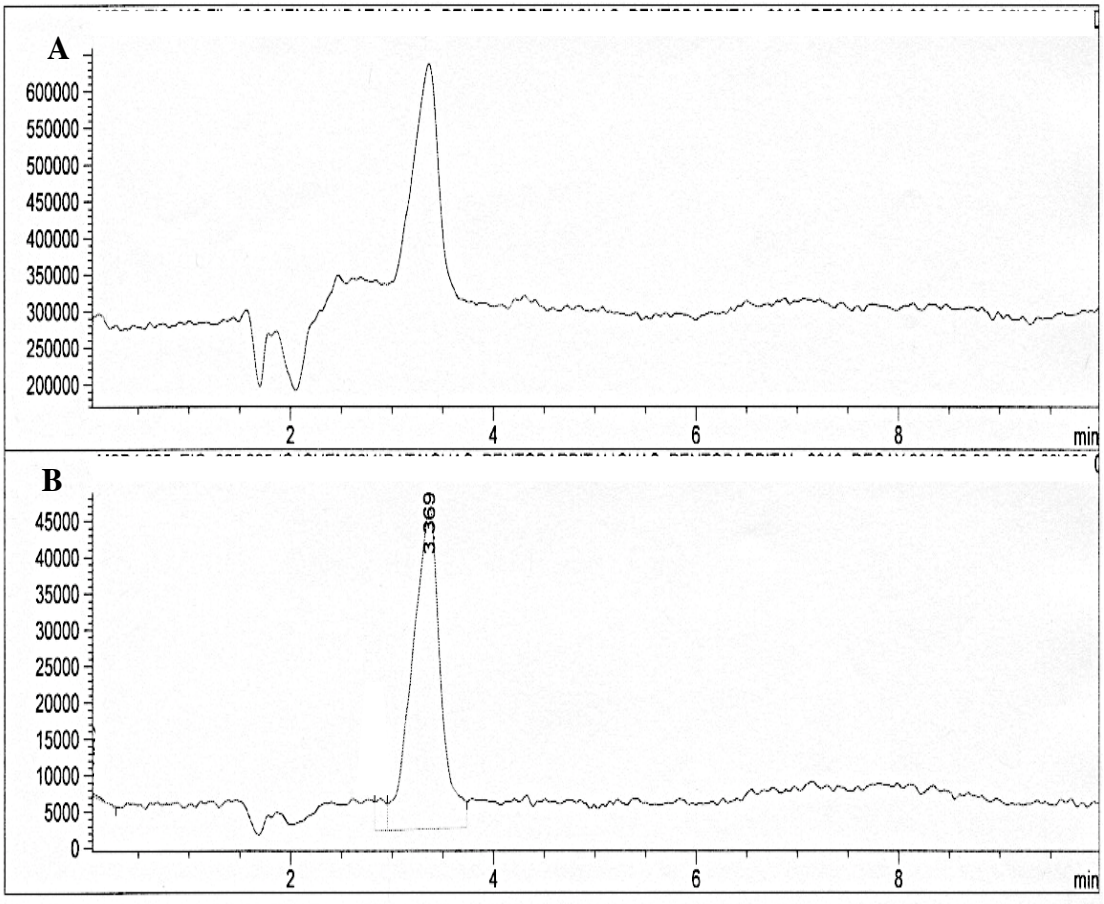


Figure 7A: Total Ion Chromatograph (TIC) of a pentobarbital standard in methanol at a concentration of 0.0002 mg/mL.

Figure 7B: Extracted Ion Chromatograph (EIC) of 225 m/z. The mass to charge ratio (m/z) of pentobarbital $[M-H]^-$ in negative mode is 225. The retention time for pentobarbital is 3.37 minutes for standards. The peak area is 8.529 x 10^5, which is significantly greater than the baseline noise of 2578.77.

The following figures illustrate the calibration curves that were established in order to test the sensitivity of the instrument for the upper and lower ranges. Calibrating the instrument was conducted regularly. The range of linearity was established using pentobarbital in methanol standards prepared as described in Chapter Two.

The first graph illustrates the upper limit of detection based on the peak area as detected by liquid chromatography / mass spectrometry versus the concentration of pentobarbital in methanol (Figure 8). The relationship begins to deviate from linearity above 0.1 mg/mL. This analysis was performed November 2011. The next graph illustrates the lower limit of detection based on the peak area as detected by liquid chromatography / mass spectrometry versus the concentration of pentobarbital in methanol (Figure 9). The instrument was able to detect as little as 0.0001 mg/mL. However, the peak was not sufficiently above the noise level to quantify. Quantification was possible at 0.0002 mg/mL. This analysis was performed in October 2012.

Finally, in the third graph, the range of linearity for the deuterated isotope pentobarbital-d_5 is illustrated (Figure 10). The deuterated isotope pentobarbital-d_5 proved to have a smaller range of detection than pentobarbital.

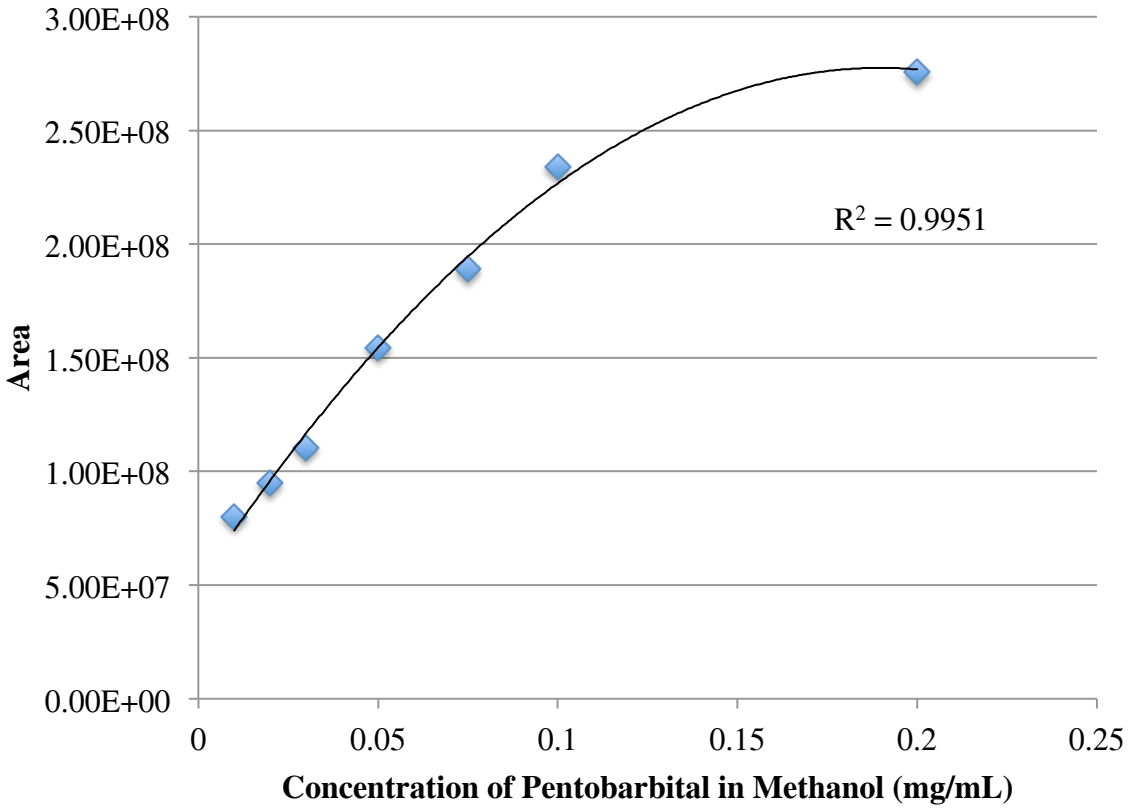


Figure 8: Calibration curve relating peak area to concentration of pentobarbital in mg/mL. Deviation from linearity occurs at 0.1 mg/mL.

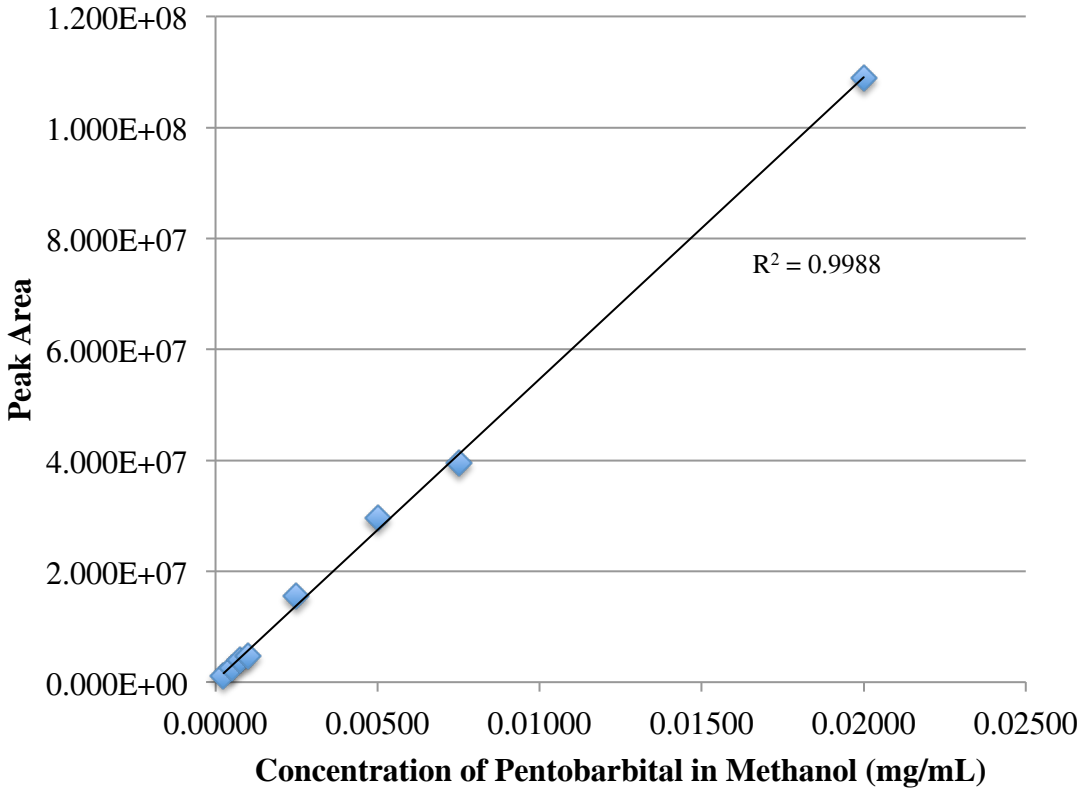


Figure 9: Calibration curve relating peak area to concentration of pentobarbital in mg/mL. Lower limit of detection occurs at 0.0001 mg/mL, lower limit of quantification occurs at 0.0002 mg/mL.

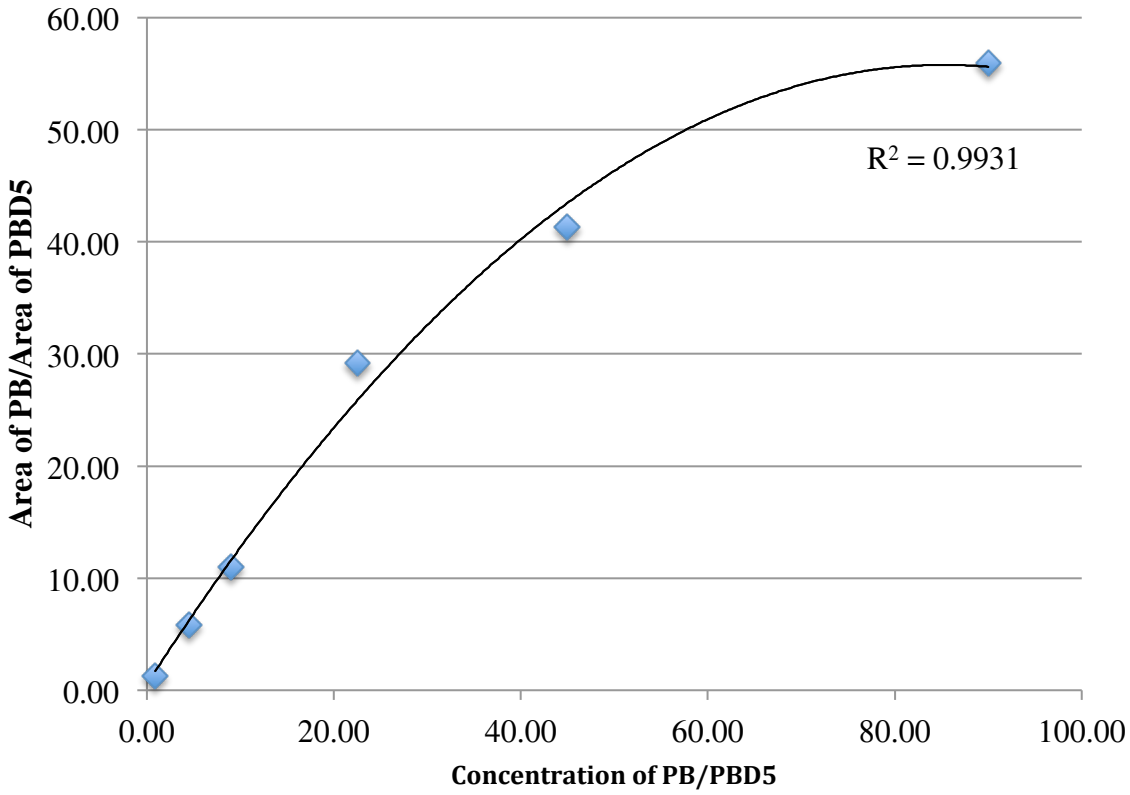


Figure 10: The graph illustrates the upper limit of detection for the internal standard pentobarbital-d_5 in methanol based on the peak area as detected by liquid chromatography / mass spectrometry. The graph shows the relationship between the ratio of the area of pentobarbital and the area of pentobarbital-d_5 versus the ratio of the concentration of pentobarbital and the concentration of pentobarbital-d_5. Deviation from linearity begins at a concentration ratio greater than 25, which equates to approximately 0.05 mg/mL of analyte, and is significant at a concentration ratio of 45, approximately equal to 0.09 mg/mL. This analysis was performed March 2012.

Based on the limits of detection, soil samples needed to contain at least 0.0005 mg and preferably 0.001 mg of pentobarbital to account for any potential loss of analyte in the methanol extraction and also in the solid phase extraction procedure and still allow the concentration of the final sample to fall within the range of detection on the LC/MS. A control of the solid phase extraction procedure was conducted to determine the efficiency at 0.001 mg in water. A 5.0 mL volume of water was added to a standard solution of 0.001 mg/mL of pentobarbital in methanol to give a final volume of 6.0 mL. The solution was then processed via the solid phase extraction procedure. A polymeric reverse phase extraction cartridge was activated and conditioned with 2 mL 5% methanol, then washed with 2 mL 0.1M sodium acetate buffer (pH 7.0). The pentobarbital sample was then passed through the cartridge and then washed with 1 mL of 0.1M sodium acetate buffer and allowed to dry under vacuum for 5 minutes. The pentobarbital was eluted from the cartridge into a fresh flask using 1.1 mL of 50:50 20% methanol/acetonitrile. The solution was then transferred to an HPLC vial for LC/MS analysis. Results of two separate analyses indicate that all of the pentobarbital was recoverable (0.001 mg) and therefore no loss of analyte should occur in this step.

It was also important to analyze soil samples to be sure they did not contain any residue of pentobarbital before spiking the samples. Unadulterated soil samples were assayed for the presence of pentobarbital. Analysis of these control soil samples failed to detect the presence of pentobarbital.

Soil samples were spiked with increasing amounts of pentobarbital solution ranging from 0.50 mL of 0.001 mg/mL to 1.0 mL of 0.10 mg/mL pentobarbital solution

to ensure the samples would be well within the LOD for verification and quantification. Samples were then processed according to the method and analyzed by LC/MS.

4.3 Results of Method Utilized with Soil

A standard calibration curve was created each day soil samples were analyzed. Soil samples included potting soil, sand, topsoil (0-10 cm), topsoil (11- 20 cm), stall sweepings, and loam. Analysis of the method was primarily with potting soil since its composition was consistent and reproducible. The method included spiking soil (5 g) with the desired concentration of pentobarbital. Extraction of pentobarbital was performed by adding 25 mL of reagent grade methanol to each prepared soil sample and mixing it overnight by automated shaking. The methanol layer was then decanted into a fresh vial and an additional 10 mL of methanol was added to the soil and shaken for 1 hour to allow the soil to settle. The methanol layer was removed and combined with the original methanol layer. The combined methanol solution was centrifuged to remove sediment and particulate matter and the supernatant was evaporated to near dryness on a rotary evaporator. Water (5 mL) was added to re-dissolve the extract and was then filtered through 0.22 μm syringe driven filter. The filtrate was then subjected to solid phase extraction to prepare it for analysis by liquid chromatography / mass spectrometry. The results of the method with pentobarbital are detailed in the following table.

Table 3: Recovery of pentobarbital from various soil types as a function of amount of pentobarbital.

Pentobarbital Soil Data			
Soil Type	**Spiked Amount (mg)**	**Recovery (%)**	**Trials**
SPE Control	0.001	103	2
Top Soil (0-10 cm)	0.001	70	1
Top Soil (11-20 cm)	0.001	111	1
Sand	0.001	87.3	3
Stall Sweepings	0.001	84.6	3
Loam	0.001	88	3
Potting Soil	0.0005	59	2
Potting Soil	0.001	85.7	8
Potting Soil	0.002	87.4	4
Potting Soil	0.004	67	3
Potting Soil	0.01	93	1
Potting Soil	0.1	103	3

The lower limit of detection for the method was 0.0005 mg/mL of pentobarbital in 5 grams of potting soil, which equates to 100 part per billion (wt/wt). At 0.001 mg per 5 grams of potting soil, the recovery was 85.7%. Percent recovery refers to the percentage of pentobarbital (mg) recovered from the soil sample as measured by LC/MS versus the amount of pentobarbital added to the soil sample. Recovery for 0.002 mg increased slightly from 85.7% to 87.4%. With the exception of the 0.004 mg trials, recovery of pentobarbital increased steadily as concentration of pentobarbital increased, peaking at 103% recovery for 0.1 mg of pentobarbital in 5 grams of soil or the equivalent of 20 ppm. The decrease at 0.004 mg was likely a result of an alteration in the solid phase extraction procedure. Water aspiration was utilized to pull the sample through the cartridge instead of a vacuum pump.

The solid phase extraction procedure (SPE) was critical in cleaning up the sample prior to LC/MS analysis. Peak area and signal to noise ratio was affected if any step of the SPE procedure was compromised. Using methanol in lieu of water allowed for faster evaporation and was preferable. Although methanol proved to be more efficient in extracting pentobarbital, it was less effective for the solid phase extraction procedure. Use of methanol caused the pentobarbital to elute prematurely. Therefore evaporating the methanol allowed the pentobarbital to be re-dissolved in water, which was much more favorable for the reverse phase solid phase extraction procedure. However, evaporating to dryness also presented some difficulties in re-dissolving the extract in water. A greater volume of water was required and thus counterproductive to the evaporation method. Further, it was noted that utilizing a strong vacuum source other than water aspiration

was more successful at drying out the extractor and increasing yield. Additionally, the buffer was key in optimizing SPE procedures and/or yield. The following figure illustrates early analysis of a soil sample processed without a buffer during the solid phase extraction method (Figure 11). The peak is barely detectable above the noise level and the mass spectrum detects multiple ions within the peak (Figure 12). Use of a 0.1M sodium acetate buffer during the solid phase extraction procedure enhanced detection of pentobarbital significantly as illustrated in the following TIC of a soil sample processed with the buffer (Figure 13). The mass spectrum of the peak also reflects a much cleaner sample (Figure 14).

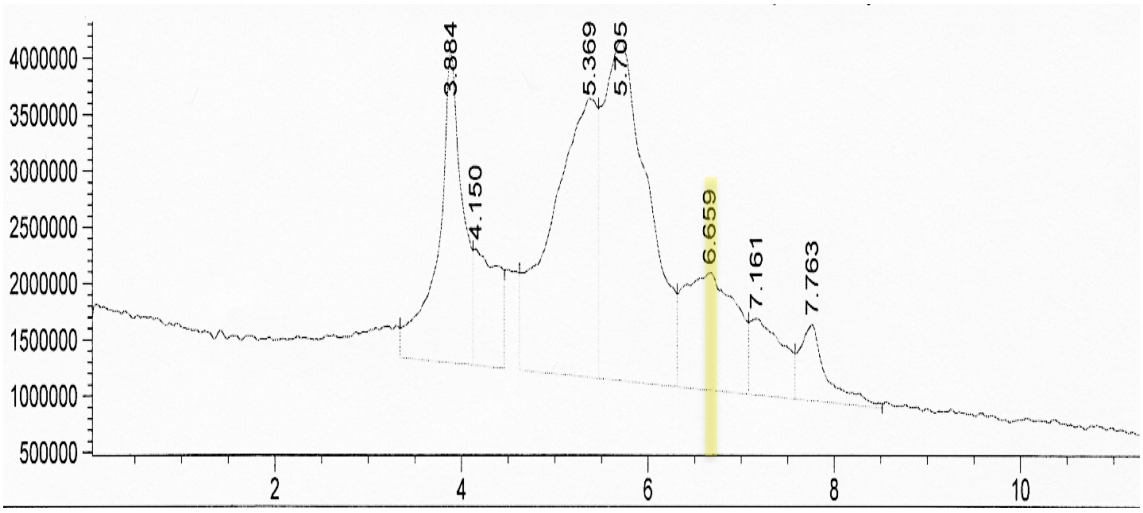


Figure 11: Total Ion Chromatograph (TIC) of a soil sample spiked with 0.1 mg of pentobarbital and processed without a buffer during SPE. Analysis performed July 2011.

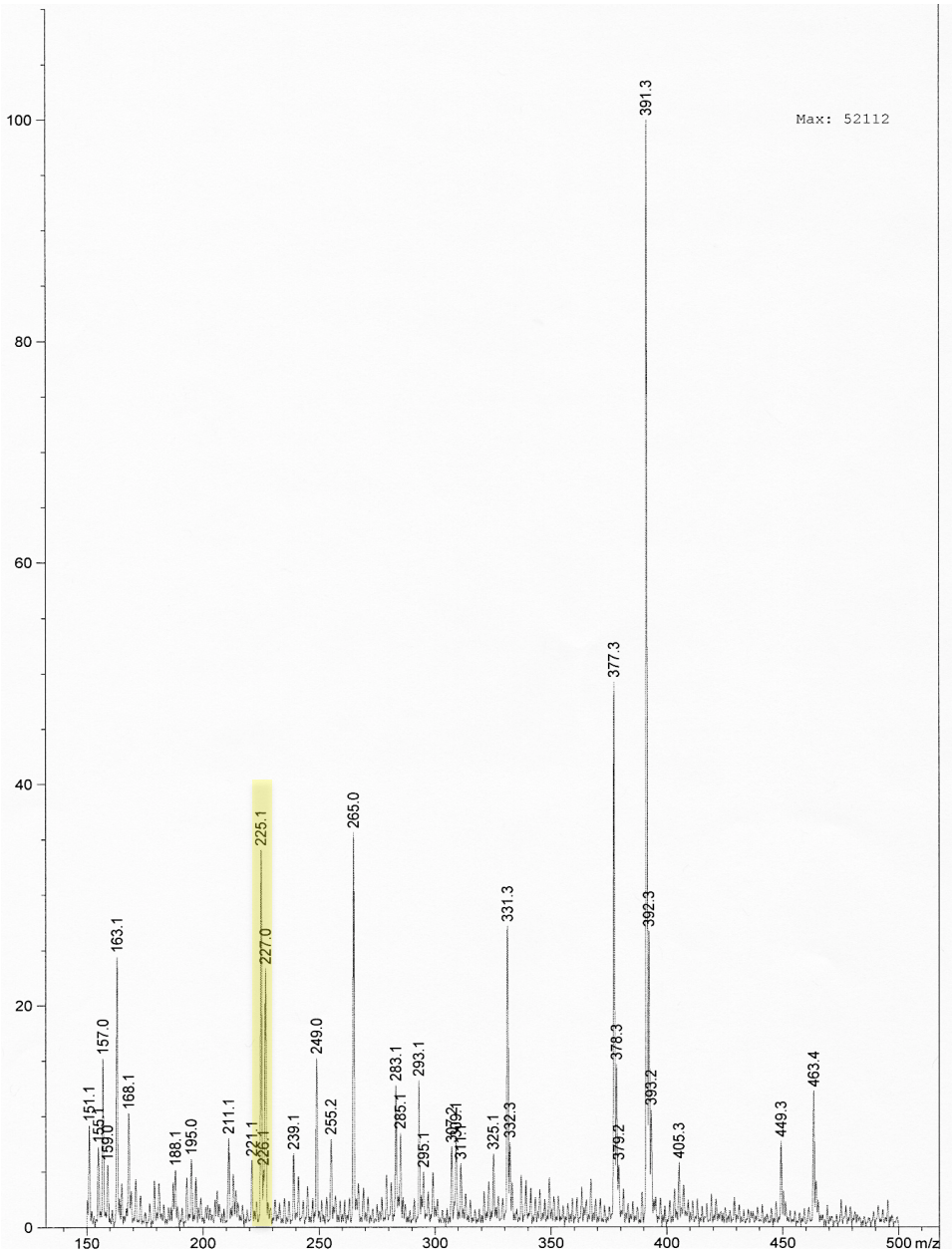


Figure 12: Ion spectra of a soil sample analyzed via LC/MS after application of the method without a buffer in the SPE procedure. Pentobarbital has a mass to charge ratio (m/z) of 225 in negative polarity mode. Analysis performed July 2011.

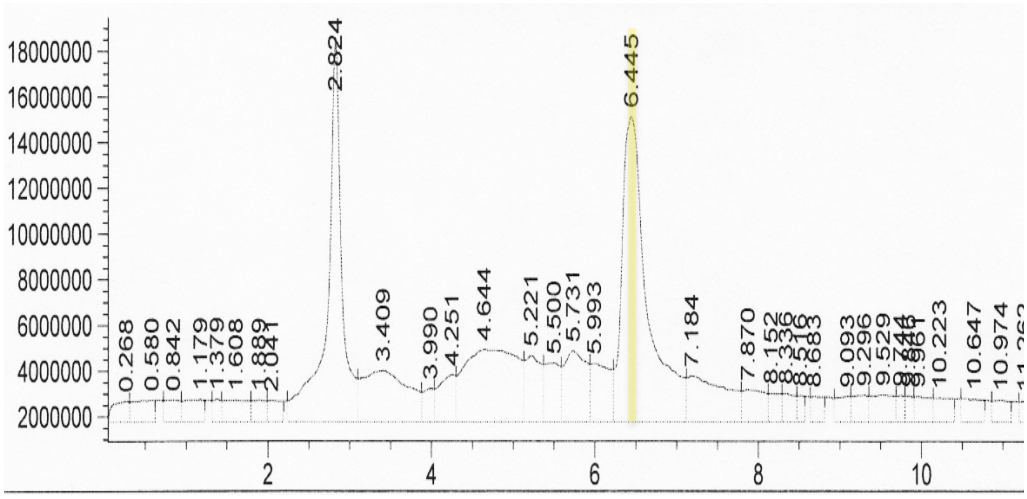


Figure 13: Total Ion Chromatograph (TIC) of a soil sample spiked with 0.1 mg of pentobarbital and processed with a buffer during SPE. Analysis performed July 2011.

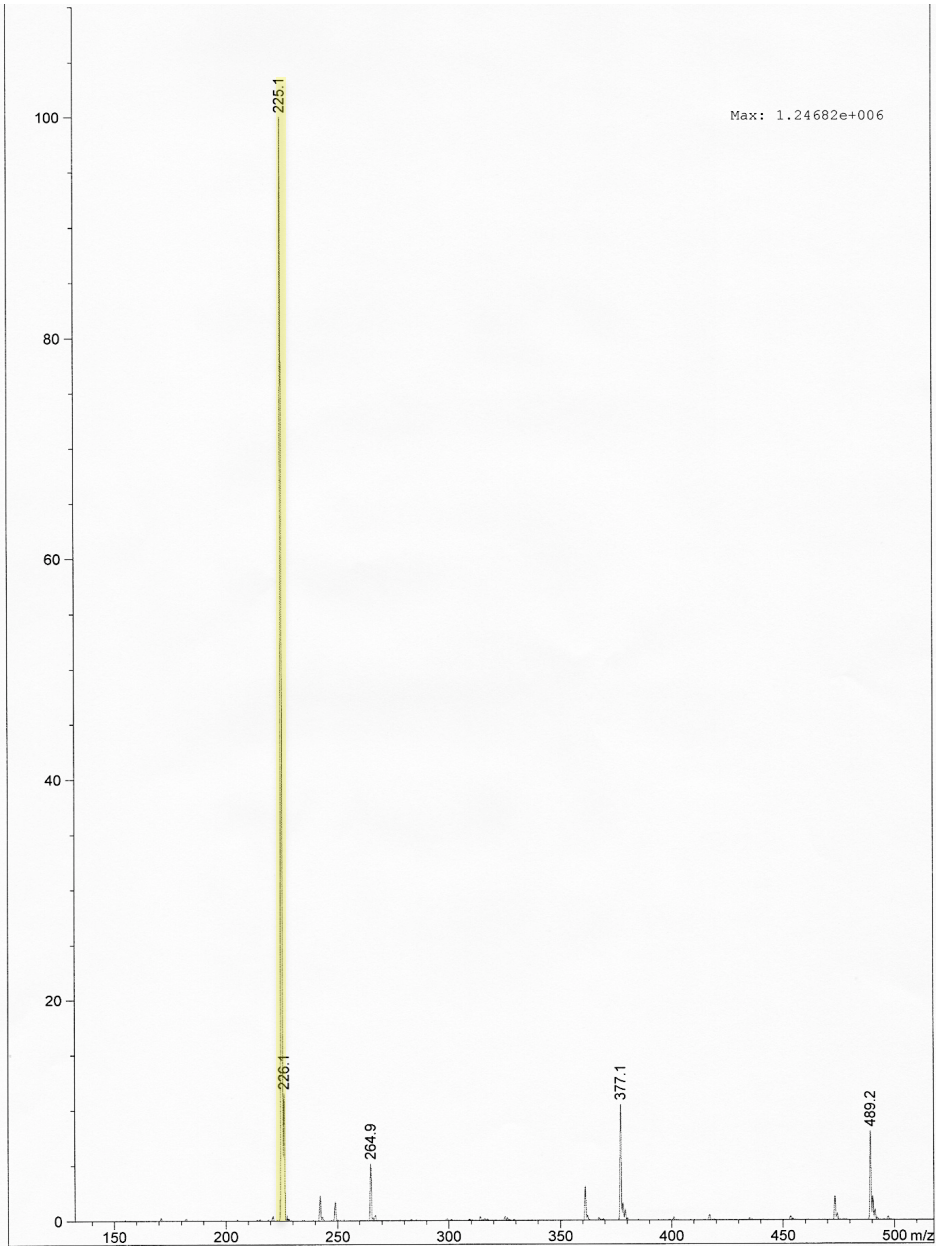


Figure 14: Ion spectra of a soil sample analyzed via LC/MS after application of the method with a buffer in the SPE procedure. Pentobarbital has a mass to charge ratio (m/z) of 225 in negative polarity mode. Analysis performed July 2011.

More over, it was important to maintain a continuous flow through the extractor until the drying step. It was imperative the extractor not dry out prematurely. Finally, to maintain a consistent volume with each sample, mobile phase was added, if necessary, to bring all samples to a final volume of 1 mL before analysis, with the exception of the internal standard trials, which were 250 μL total volume.

Generally, retention times were equally consistent from one day to the next with the variation not exceeding 0.1 minutes. Although, the peak area was consistently above the noise level, variations in peak area could be profound from one week to another. A calibration curve was established each day analysis was conducted and multiple analyses of the same sample were conducted on different days to confirm reproducibility and consistency of recovery. Recovery rates followed an increasing trend for increasing concentrations with one exception, the 0.004 mg samples.

4.4 Internal Standard Results

An internal standard is an alternative method of quantitative analysis that is effective when slight variations in the instrument response occur from run to run and are difficult to control (Harris, 2009). Internal standards also account for variations in physical parameters such as microinjection volumes or pipetting technique (Christian, 2004). This method involves spiking the sample with an equal amount of a solute with similar chemical properties and a retention time near to the retention time of the analyte of interest. The ratio of the area of the analyte to the area of the internal standard versus the amount of analyte is then used to construct the calibration curve. The use of a

deuterated isotope as an internal standard is often considered an ideal choice when possible. Isotopes are atoms of the same element but with different mass numbers. The simplest example is hydrogen. A deuterium atom, or ^{2}H, is simply a hydrogen atom with one proton and one neutron instead of one proton as with the common form of hydrogen, or ^{1}H. The molecular mass of deuterium is 2.014 grams per mole versus the 1.007 grams per mole of ^{1}H. Similarly, deuterated compounds are compounds that contain some deuterium atoms in place of hydrogen atoms. Deuterated compounds are ideal internal standards due to the near identical nature of the deuterated compound to that of the analyte of interest. A minimum of three to five deuterium atoms are generally considered ideal to serve as an internal standard or deuterated isotope standard so that the molecular weight is easily distinguishable utilizing mass spectrometer.

Results of soil analysis with the deuterated isotope pentobarbital-d_5 were comparable to analysis utilizing the external calibration method. A 25.0 µL volume of pentobarbital-d_5 was added to 225 µL extract of the processed soil samples and analyzed by liquid chromatography / mass spectrometry under the same operating conditions as previously detailed. Results were then subjected to the F-Test, which is designed to indicate if there is a significant difference between two methods based on their standard deviation and the variances between two methods. If a calculated value exceeds the tabulated value, then there is a statistical significant difference in the variances. However, if the calculated value is less than the tabulated value, a significant difference in methods cannot be determined via the F-Test and a T-test can be performed. The T-test is often used to measure the difference between two methods based on the results. If the

calculated *t* value is greater than the tabulated value for a specific confidence level, then the results are statistically different. If the calculated *t* value is less than the tabulated value at a specific confidence level then the results are statistically similar and the two methods analyzed gave statistically similar results (Christian, 2004).

After analyzing the samples and computing the data, the F-test revealed a value of 10.03 versus the tabulated value of 10.97. Therefore, the hypothesis of statistically similar variances could not be rejected. A T-test was performed to assess the difference between the means. The T-test revealed a value of 2.03, which is less than the T-table value of 2.57 leading to the conclusion that the two data sets gave statistically significant similarities. As a result, much of the subsequent analyses were conducted using an external calibration method. This method was significantly faster and cost effective. The results of the analysis are detailed in the following two graphs (Figure 15 and 16).

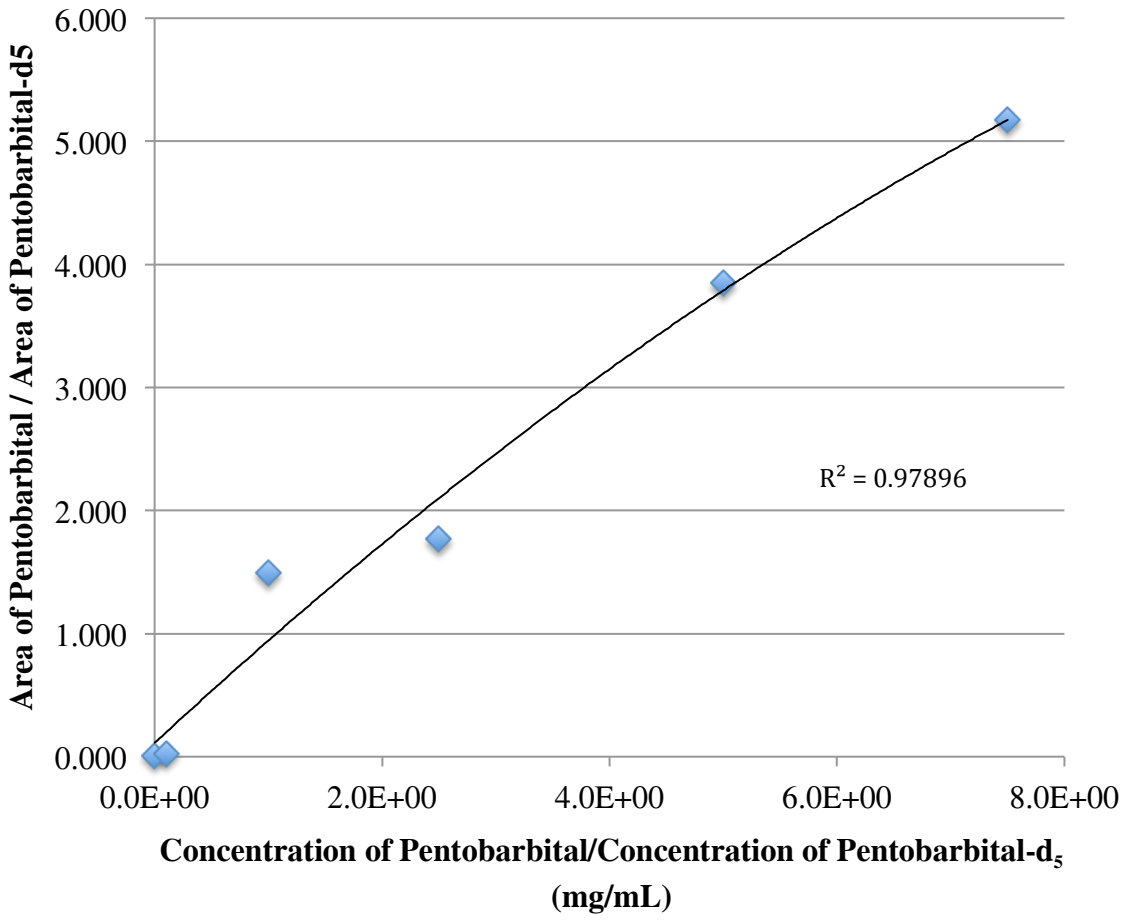


Figure 15: The graph illustrates the calibration curve established for the internal standard pentobarbital-d_5 in methanol and pentobarbital based on the peak area as detected by liquid chromatography / mass spectrometry. The graph shows the relationship between the ratio of the area of pentobarbital and the area of pentobarbital-d_5 versus the ratio of the concentration of pentobarbital and the concentration of pentobarbital-d_5. This analysis was performed in February of 2012.

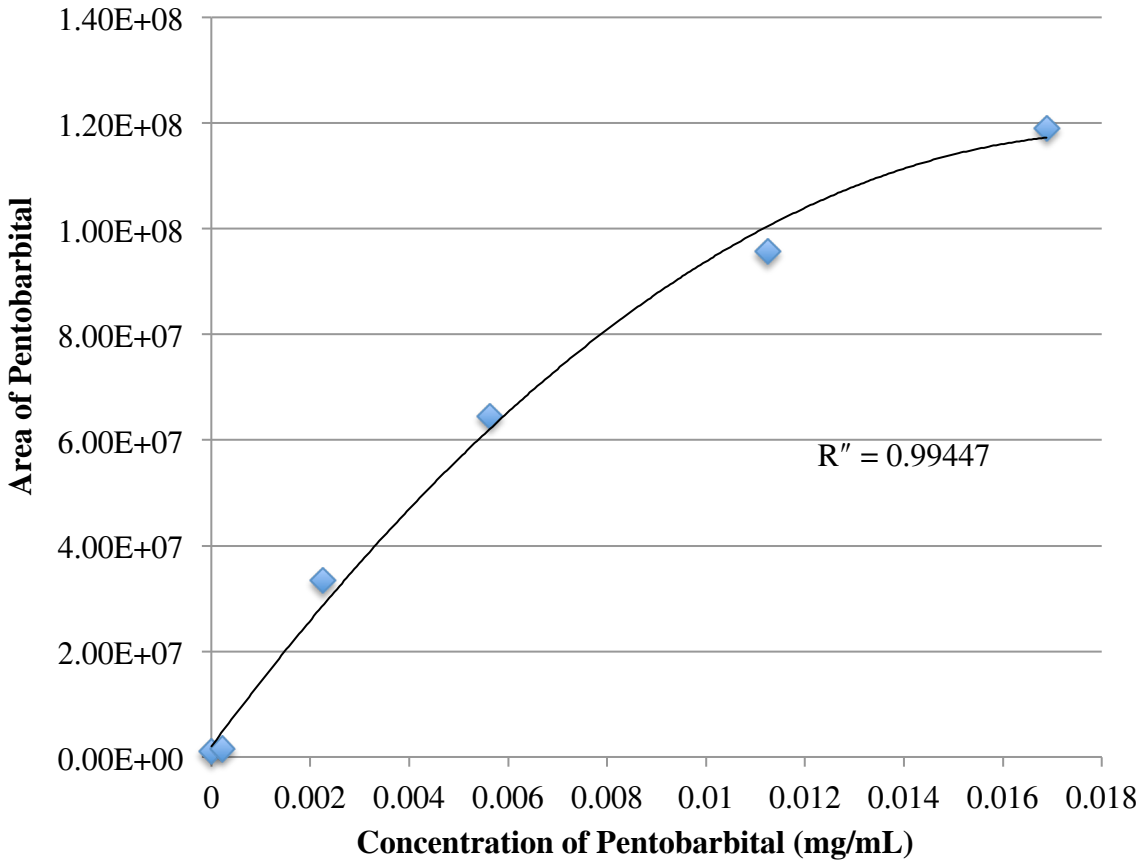


Figure 16: The graph illustrates an external calibration curve established for pentobarbital in methanol as detected by liquid chromatography / mass spectrometry. The graph shows the relationship between the area of the peak for pentobarbital and the concentration of pentobarbital. This analysis was performed in February of 2012.

The table below details the F-test and T-test performed to analyze the variances and results, respectively, between using a deuterated isotope calibration versus an external calibration of pentobarbital of six soil samples processed via the method for detecting pentobarbital in soil by solid phase extraction and LC/MS (Table 4). Method 1 was an analysis of the detection method with the deuterated isotope pentobarbital-d_5 (PBD5) as an internal standard. Method 2 was an analysis of the detection method with an external calibration using pentobarbital (PB).

After comparing the two methods, there was no significant difference in variances or results. Therefore, the external calibration method was selected as the primary method for quantitative analysis due the ease of use, time efficiency, and cost effectiveness of pentobarbital as compared to pentobarbital-d_5.

Table 4: The table below details the F-test and T-test performed to analyze the variances and results, respectively, between using a deuterated isotope calibration versus an external calibration of pentobarbital of six soil samples processed via the method.

	Method 1 PBD5	**Method 2 PB**	**F-Test and T-Test for Two Methods**		
Soil samples	Percent Recovery			Method 1	Method 2
1	9	8	Mean	25.32	10.28
2	12	8	Variance	149.21	14.88
3	30	15	Observations (n)	6	6
4	25	14	Pooled Variance	12.81	
5	40	5	Degrees of freedom	5	
6	35	11	**T-Stat** (calculated)	**2.034**	
			P(T<=t) one-tail	0.008	
			P(T<=t) two-tail	0.016	
Average	25.3	10.3	T Critical two-tail (T test table)	**2.571**	
Standard Deviation	12.2	3.9	Confidence interval at 95%	15.17	4.79
Sum of the square of differences	746.1	74.4	Correlation coefficient	0.065	
Variance	149.2	14.9	**F-Test** (calculated)	**10.029**	

4.5 Long Term Recovery of Pentobarbital in Soil

An analysis of the possible decay of pentobarbital in soil was conducted over a 17-week period. Three 35 gram samples of potting soil, sand and topsoil (11-20 cm) respectively were each spiked with 0.07 mg of pentobarbital and mixed thoroughly. The samples were each divided into seven 5 g samples and stored in a 50 mL centrifuge tube and incubated at 37 ºC over a 17-week period of sampling. At approximately 4-week intervals, one sample of each soil type was removed from incubation and analyzed via the method for pentobarbital (mg). The amount of pentobarbital (mg) that persisted in soil over the 17 weeks is detailed below (Table 5).

After analysis of the data, it appears as though soil characteristics may affect total recovery. Although potting soil typically has a pH in the range of 6.0 – 6.8 to support healthy plant growth, the Expert Gardner All Purpose Potting Soil mix utilized in this study had a pH of 7.0. The pKa of pentobarbital is reported to be 7.8, suggesting that it exists in the environment in an anion form. Although pentobarbital does not readily sorb to soils and specifically alkaline soils, it was contained in a vial that did not permit it to be carried away by ground water. The possibility that the pentobarbital sorbed to the soil over time must be considered. Microbial degradation could also be a possibility, however it may be impeded if the pentobarbital was bound to the soil. Based on the long term recovery data of the potting soil samples, it would suggest that pentobarbital was subject to degradation by microbial influences or bound to the soil or possibly both. Based on successful recoveries of pentobarbital in the analysis of soil types and recovery rates (Table 3) in the first study, it would suggest that soil equilibrium may play a part in the

ability to recover pentobarbital over time. Based on the stability of pentobarbital and the limited biodegradation data available, it is likely degraded by microbial influences is minimal. In the first study, pentobarbital had a recovery of 93% for potting soil. At the same concentration, the 4-week decay sample had a recovery of only 34.1%.

Over the 17-week period of analysis, the recovery declined to 10.0% for potting soil. Based on comparative data, it appears as though pentobarbital may be approximately 83% unrecoverable. Interestingly, the long term recovery of pentobarbital had a significant decline after week 8.

Similarly, the long term recovery of pentobarbital in sand and topsoil was also compromised. Sand had a recovery of 37.2% for the 4-week sample and a recovery of 17.0% for week 17. In the first study, sand had a recovery of 87.3% for 0.001 mg of pentobarbital in sand. At a 10-fold increase in concentration, recoveries would be expected to increase based on the trend (Table 3). However, recovery decreased significantly. Topsoil had a recovery of 38.3% for the 4-week sample and a 19.3% recovery for the 17-week sample. In the first study, all the pentobarbital was recovered at 0.001 mg. The unrecoverable amount of pentobarbital based on the decrease in recovery is at least 80%. The decline in recoveries was consistent for all soil types tested. In the following table (Table 5), the results of the 17-week study are detailed. The long term recovery of pentobarbital may be affected by exposure to soil or possibly even degradation. If long term exposure to soils allows degradation to occur, this would call into question the stable nature of pentobarbital.

Table 5: Long term recovery of pentobarbital in soil.

Samples	Date of Analysis	Period of Analysis	% Recovery[a]	Calculated Concentration (mg)[b]
Potting soil	12/19/12	4 weeks	34.1	0.0034
Potting soil	01/18/13	8 weeks	29.5	0.0030
Potting soil	02/04/13	11 weeks	10.4	0.0010
Potting soil	03/19/13	17 weeks	10.0	0.0010
Sand	12/19/12	4 weeks	37.2	0.0037
Sand	01/18/13	8 weeks	38.1	0.0038
Sand	02/04/13	11 weeks	22.5	0.0023
Sand	03/19/13	17 weeks	17.0	0.0017
Topsoil	12/19/12	4 weeks	38.3	0.0038
Topsoil	01/18/13	8 weeks	40.7	0.0041
Topsoil	02/04/13	11 weeks	19.6	0.0020
Topsoil	03/19/13	17 weeks	19.3	0.0019

[a]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.

[b]Calculated concentration was the amount of pentobarbital (mg) calculated to be in the soil sample tested as determined by an external calibration method.

4.6 Dispersion of Pentobarbital in Soil

A 5 g sample of potting soil was spiked with 1 mg of pentobarbital sodium salt packed into a 10 mL syringe. This resulted in a column of soil 7.5 cm tall. Three 10 mL volumes of distilled water were pulled through the column of soil under vacuum. Each of the three filtrates were collected separately and analyzed for pentobarbital. The soil within the column was then separated into three layers, the top layer consisting of the first cm, the second layer the next three cm, and the third layer containing the last 3.75 cm. Each of these layers of soil was analyzed for pentobarbital via the method. The amount of pentobarbital within the first column volume of water had the greatest amount of pentobarbital among all the soil and water samples as determined by area under the curve analyzed by LC/MS. It contained 54.5% of the total pentobarbital (mg) recovered. The first layer of soil had the greatest concentration of pentobarbital per cm of soil at 2.5%. However, pentobarbital was distributed fairly evenly throughout each of the soil layers with percentages of distribution at 2.5%, 2.3% and 2.6% for the three soil layers.

Dispersion results suggest pentobarbital in soil readily contaminates ground water and evenly distributes throughout the soil as it is carried via the water supply (Table 6).

Table 6: The dispersion of pentobarbital in soil and water. Pentobarbital is highly mobile in water and showed the greatest concentration in the first column volume of water. The top layer of soil contained the highest concentration of pentobarbital of the three layers. The pentobarbital was loaded onto the top layer.

Dispersion of Pentobarbital		
Sample	Area Under Peak	Percent of Total
1st column volume of water (10% of total water was analyzed)	1.16×10^{8}	54.5%
2nd column volume of water (10% of total water was analyzed)	4.90×10^{7}	23.0%
3rd column volume of water (10% of total water was analyzed)	3.22×10^{7}	15.1%
0-1 cm soil	5.34×10^{7}	2.5%
1-4 cm soil	4.91×10^{7}	2.3%
4-7.5 cm soil	5.43×10^{7}	2.6%

4.7 Breakdown of Pentobarbital by Bacteria

The decay of pentobarbital by various strains of gram negative bacilli, including but not limited to W2B, HSC-A, HSC-D, and alpha small, was analyzed to determine which strain had the most potential for degrading pentobarbital. Initial analysis conducted by the Department of Biology at Middle Tennessee State University via enzyme linked immunosorbent assay (ELISA) and optical density measurements indicated W2B, HSC-A, HSC-D, and alpha small were possible candidates for possessing the enzyme barbiturase that could degrade the barbiturate pentobarbital. Of the four samples, the alpha small strain appeared to have the most success in breaking down pentobarbital based on initial crude analyses. Supernatant, from each of the four bacteria, was analyzed using liquid chromatography / mass spectrometry according to the method described in this thesis to assess the best candidate for future research.

Supernatant was collected from the bacteria each day for a period of one week. A 1 mL aliquot of the supernatant from the bacteria samples was diluted to 5 mL with 18Ω water and filtered through a Millex – GV PVDF 0.22 μm syringe driven filter and then subjected to solid phase extraction and analyzed by liquid chromatography / mass spectrometry according to method parameters.

The degradation of pentobarbital by the bacterium, HSC-A, W2B and HSC-D could not be confirmed via LC/MS. There was no significant decrease in concentration of pentobarbital as measured by the peak area (Table 7). Results for alpha small were inconclusive and therefore, the possibility that this bacterium may contain an enzyme capable of metabolizing pentobarbital could not be rejected (Table 8) .

Table 7: Change in pentobarbital concentration according to change in peak area as measured by LC/MS for various strains of bacteria.

Date	Bacteria	Growth Media	Hours since inoculation	Peak Area
7/20/2011	W2B Supernatant, SPE	Minimal Broth	24	1.32E+08
7/20/2011	W2B Supernatant, SPE	Minimal Broth	72	1.38E+08
9/16/2011	W2B Supernatant, SPE	Minimal Broth	24	1.41E+08
9/16/2011	W2B Supernatant, SPE	Minimal Broth	168	1.94E+08
9/1/2011	HSC-A Supernatant, SPE	Minimal Broth	24	**2.97E+08**
9/1/2011	HSC-A Supernatant, SPE	Minimal Broth	168	**2.58E+08**
9/2/2011	HSC-A Supernatant, SPE	Minimal Broth	24	1.65E+07
9/2/2011	HSC-A Supernatant, SPE	Minimal Broth	168	2.66E+07
9/15/2011	HSC-D Supernatant, SPE	Minimal Broth	24	1.35E+08
9/15/2011	HSC-D Supernatant, SPE	Minimal Broth	168	1.41E+08
9/15/2011	HSC-D Supernatant, SPE	Minimal Broth	24	1.71E+08
9/15/2011	HSC-D Supernatant, SPE	Minimal Broth	168	2.08E+08

Table 8: Change in pentobarbital according to change in peak area for alpha small. Bold type indicates a decrease in area as measured by LC/MS, correlating to a decrease in concentration of pentobarbital. Preliminary ELISA and optical density analysis indicated this strain had the most potential. Quantitative analysis via the LC/MS did not support significant degradation of pentobarbital.

Date	Bacteria	Solution	Hours since inoculation	Peak Area
7/28/2011	Alpha Supernatant, SPE	Minimal Broth	24	8.56E+07
7/28/2011	Alpha Supernatant, SPE	Minimal Broth	72	9.69E+07
8/2/2011	Alpha Supernatant, SPE	Minimal Broth	24	1.45E+08
8/2/2011	Alpha Supernatant, SPE	Minimal Broth	72	1.50E+08
9/16/2011	Alpha Supernatant, SPE	Minimal Broth	24	**1.81E+08**
9/16/2011	Alpha Supernatant, SPE	Minimal Broth	168	**1.78E+07**
2/3/2012	Alpha Supernatant, SPE	Minimal Broth	24	**4.83E+07**
2/4/2012	Alpha Supernatant, SPE	Minimal Broth	96	**3.59E+07**
2/17/2012	Alpha Supernatant, SPE	Minimal Broth	24	8.92E+07
2/17/2012	Alpha Supernatant, SPE	Minimal Broth	168	1.06E+08
6/13/2012	Alpha Supernatant, SPE	Minimal Broth	24	2.22E+08
6/14/2012	Alpha Supernatant, SPE	Minimal Broth	168	2.32E+08

Further analysis of alpha small was conducted to determine the viability of the presence of an enzyme capable of degrading pentobarbital. Protein concentration was assayed to determine the presence of protein. Results were positive for protein. Further analysis was conducted to measure for enzyme activity. A liter solution minimal broth with pentobarbital and zinc chloride was inoculated with alpha small cells and placed in an incubator at 37ºC. After a period of days, the solution was centrifuged and the supernatant poured off. The remaining pellet of cells was then resuspended in a 0.2mM potassium phosphate buffer solution (pH 7.0) and subjected to sonification or bead beater to break open the cells. The solution was then centrifuged at 10,000 RCF (relative centrifugal force) to eliminate the cellular debris. A 20 μL volume of 0.1mM solution of pentobarbital was added to an aliquot of the alpha small supernatant and analyzed for kinetic activity on a Hitachi U2900 UV spectrophotometer at 256 nm for 20 minutes. There was no change in the absorbance. Results were negative for activity, consistent with analysis of supernatant.

CHAPTER V

CONCLUSION

In summary, the stable structure of pentobarbital, the additive effects with other pharmaceuticals, and its continued usage has led to an increasing environmental hazard. Pentobarbital is leaching into soil, water, and even the food supply. This concern is prompting researchers to develop methods of detection in complex matrices and understand factors affecting the degradation of these drugs so that viable alternatives or solutions for protecting our environment and our health can be achieved. SPE in concert with LC/MS proved to be an effective method for detecting and quantifying these drugs in soil. The LC/MS was successful in detecting pentobarbital in soil at a concentration of 0.2 μg of pentobarbital per gram of soil. The LC/MS allows for a relatively quick assessment of a sample without the added step of derivatization necessary for GC/MS. Further research would be prudent in determining if this method is effective for all barbiturates in soil, water or other complex matrices. Research in the area of pentobarbital stability and degradation is currently under review. Long term recovery data suggests that pentobarbital may not be as stable as expected in all matrices. Although pentobarbital resists degradation in water, the decreased recovery after long term exposure to soil suggests that either decay or interactions with the soil is occurring.

A thorough analysis of the parameters of the LC/MS was carried out before proceeding with the quantitative analysis of pentobarbital in soil. LC/MS analysis was effective in detecting and quantifying pentobarbital in methanol at a minimum

concentration of 0.0002 mg/mL. When added to soil, 0.0005 mg of pentobarbital in 5 grams of soil could be detected. Even as the concentration approached the lower limits of detection for the method, recovery rates were well within expected limits at 85.7% for 0.001 mg of pentobarbital in 5 g of potting soil. All the pentobarbital was recovered at a concentration of 0.1 mg of pentobarbital in 5 g of potting soil. Various soil types including topsoil (0-10 cm), topsoil (11-20 cm), stall sweepings, loam and sand were analyzed for recovery of pentobarbital. Differing soil types had little effect on percent recovery. All soil types tested had modest recoveries at 0.001 mg of pentobarbital in 5 g of soil.

Pentobarbital is considered to be a highly stable compound and unavailable for degradation in soil due to its high degree of mobility in water and poor sorption to soils. Results of the long term recovery of pentobarbital in soil suggest pentobarbital does decay or bind to the soil. A steady decrease in pentobarbital concentration in soil occurred over the 17 weeks with the greatest change occurring between weeks 8 and 11. A more gradual rate of decay occurred over the remaining 6 weeks. It is suggestive that pentobarbital does decay if it sorbed into soil or was prevented from migrating into ground water.

The dispersion of pentobarbital in soil and water was analyzed and results showed the greatest concentration of pentobarbital was in the first column volume of water, indicating that pentobarbital in soil readily contaminates ground water. Further, minute amounts of pentobarbital were evenly distributed throughout the soil as it is carried along via the water supply.

Analysis of the decay of pentobarbital by soil bacterium was also analyzed in this research. Although early indications of a gram-negative bacillus strain appeared positive for the barbiturase enzyme capable of degrading pentobarbital, quantitative analysis by LC/MS and enzyme kinetic analysis could not confirm the degradation of pentobarbital.

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