ANALYSIS OF BIOGENIC AMINES IN VITREOUS HUMOR USING HIGH
PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ESTIMATION OF
POSTMORTEM INTERVAL

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LIST OF ABBREVIATIONS

IS: Internal Standard

HPLC: High performance Liquid Chromatography

NP: Normal Phase

PMI: Post-Mortem Interval

RP: Reverse Phase

UV/VIS: Ultraviolet-Visible Spectrophotometry

ABSTRACT

ANALYSIS OF BIOGENIC AMINES IN VITREOUS HUMOR USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ESTIMATION OF POSTMORTEM INTERVAL

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A novel method is proposed to estimate the postmortem interval (PMI) using the biogenic amine concentration in vitreous humor. There are multiple methods that can be used to determine the time of death (PMI). However, these methods often have uncertainties that complicate estimating an accurate PMI. To address this challenge and improve the accuracy in PMI determination, this study focuses on utilizing biogenic amine levels in vitreous humor. Biogenic amines, formed from the decarboxylation of proteins during anaerobic bacterial degradation following discontinuation of oxygen transport at death, exhibit increased concentrations in body tissues and surrounding areas. Vitreous humor is a valuable specimen collected during the autopsies for the analysis of various drugs and metabolites. It is an anatomically isolated specimen and crucial information on the presence of drugs such as alcohol and other substances at the time of death due to the lack of enzymes. The formation of biogenic amines in vitreous humor may occur at a slower rate compared to other tissues in the body and thus this matrix can provide more accurate information on PMI. A high-performance liquid chromatographic (HPLC) method was developed to analyze biogenic amines in vitreous humor as a part of this thesis research. Baseline separation of amines of interest including methylamine, cadaverine, putrescine, histamine, tyramine, and heptylamine was achieved. For a more accurate representation of the biogenic amine concentrations 1,7-diaminoheptane was used as an internal standard to adjust for any matrix effects.

Porcine eyes were chosen to be representative of human eyes due to the similarities in vitreous humor volume and composition. The eyes were let to decompose under three different environmental conditions to evaluate the impact environmental conditions have on the PMI. One interset of eyes was placed in polyethylene bags after the collection and let to decompose at room temperature. The second set of eyes were frozen for three days before the decomposition in polyethylene bags. The third set of eyes were placed in a large polyethylene box and let to decompose at room temperature. In these three experiments analysis indicated that methylamine, putrescine cadaverine, and histamine are present in the vitreous humor. Concentrations of methylamine, putrescine, cadaverine, and histamine are best represented in the frozen eye experiment to estimate PMI. Additionally, eyes then were put directly into the sealed bag had predictable concentration patterns of methylamine, putrescine, and cadaverine. The results from the third experiment (boxed eyes) were inconclusive as they did not have any predictable concentration correlation over time particularly for cadaverine and histamine, therefore, should not be used for PMI estimation.

CHAPTER 1: INTRODUCTION

1.1 Postmortem Interval (PMI)

A variety of methods are employed in forensic investigations to determine the postmortem interval (PMI) or the time since death. These methods can be divided into two main categories based on the stage of decomposition. Early PMI methods used mostly by pathologists based on the postmortem changes or stages of death including rigor mortis, algor mortis, and livor mortis can only be used within 48-72 hours after death. Bodies that have been decomposing for extended periods of time utilize long term PMI by determining the stage of skeletonization. These methods include radiocarbon dating, analysis of other radioisotopes such as selenium, phosphorus and radium, fluorescence-based techniques, bone extracellular matrix component analysis, proteomics of bones, radiology and spectroscopy based methods utilize bones of the deceased. Additionally, taphonomy analysis and morphology, and entomological activity of the deceased body was also used to estimate PMI.[1]

Unfortunately, PMI cannot be determined with certainty, especially for bodies that have begun to skeletonize. Using multiple methods can be beneficial in decreasing the PMI to get a better estimate of the time of death. Bodies that recently began to decompose utilize analysis methods that have PMI estimates that are more accurate to the time of death than the PMI methods that are used for cadavers that have been decaying for extended periods of time. Analysis begins by looking at the stages of death. [2]

Rigor mortis occurs immediately after death when muscles begin to become flaccid, they begin to stiffen and freeze one to three hours later. The body will remain stiff until rigor passes or

is physically broken. Rigor is considered complete when the jaw, elbow and knee joints are immoveable. The rigor process takes about 10-12 hours to develop. The body will remain stiff for 24 to 36 hours then loosen in the same order it the stiffened. Much like many of the other methods to determine PMI, there are numerous factors that impact the course of rigor mortis. This includes environmental and internal body temperatures, body composition, and activity before death. [2]

The next stage of death is livor mortis. This is the discoloration of the body after death. Approximately an hour after death, there is gravitational settling of the blood since it is no longer being pumped throughout the body, referred to a blood pooling. Within 8-10 hours, the blood pooling becomes fixed and increases in intensity until then. The location of the discoloration as a result of blood pooling may also indicate the position of the body during death. In addition to blood pooling, a violet or purple form of discoloration may appear on lighter skinned individuals known as lividity. Blood pooling and lividity can be used to determine if certain poisons were in the system and if the body had been refrigerated based on the color. Livor mortis can be visible up until the body decomposes. The coloration caused by livor mortis can easily be mistaken or conceal contusions on the body. To determine the difference an incision must be made. Lividity will have blood in the blood vessels, whereas contusions have blood within the soft tissue. Decomposition will make analysis of livor mortis difficult.[2]

The last stage of death is algor mortis or "body cooling." This is the stage when the body equilibrates with the surrounding environment. Due to numerous effects in postmortem cooling, algor mortis cannot be an accurate representation of PMI, but can be most useful with the first 10-12 hours when in a cool environment, 21-23 $^{\circ}$C. Under these conditions, a body can be projected to decrease temperature at a rate of approximately -16.94 $^{\circ}$C per hour, but as stated before, can be easily affected by many factors such as towards the end of the cooling interval, the cooling rate

slows down, the insulation on the body (fat, clothing) and the rate the temperature decreases is assuming the temperature of the body during death is 37.06 °C. If a body is in an environment of a severe freeze and was dead long enough to undergo a deep freeze, once thawed the decomposition will begin rapidly. For obtaining the PMI, the body temperature and environmental temperature should be taken as soon as possible. The temperature should be taken at least twice every hour. [2]

Most of these methods are highly subjective and have no chemical basis that can be measured. One method of determining the PMI is analyzing the potassium concentration $[K^+]$ in the vitreous humor. This method was introduced more than 40 years ago and has been recommended as a chemical method for the determination of PMI. The relationship between $[K^+]$ and PMI expresses a linear relationship and produces the linear regression for up to 133 hours postmortem. Although this method has wide range of error (about ± 24 hours), it is beneficial since a PMI estimation can be decreased to several days after death.[3]

1.2 Vitreous Humor

The vitreous humor is a transparent gel liquid inside the eyeball that fills the space between the lens and the retina. A diagram of the cross section of the eye is shown in Figure 1. The vitreous humor is composed of 98% water, hyaluronic acid, and collagen fibrils. There also is an absence of blood vessels as it is anatomically isolated from the body.[4] Since the vitreous humor is compartmentalized, it can be easily removed and is less subject to postmortem changes. Additionally, vitreous humor can be present when other biological fluids are not present.[5] These criteria make the vitreous humor a good candidate for PMI estimation.
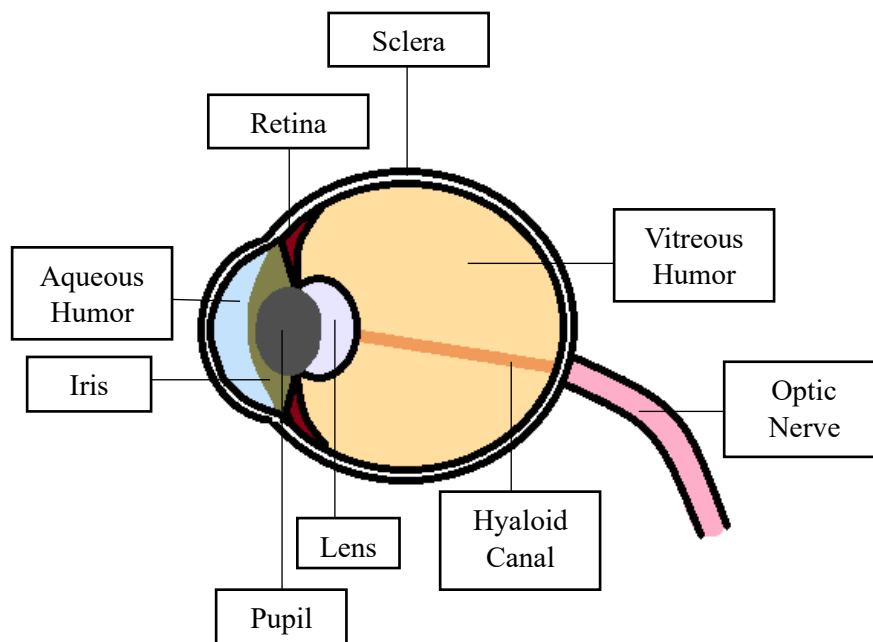
Figure 1. Cross Section of the Eye.

1.3 Biogenic Amines

Biogenic amines are low molecular weight organic bases of aliphatic, aromatic, or heterocyclic structures that are formed as a result of the decarboxylation of free amino acids. Biogenic amines are present in the body at low concentrations, which help a variety of bodily functions such as cell proliferation and differentiation, regulation of nucleic acids, protein synthesis, nerve growth and regeneration, and brain development. They can also be found in food and beverages.[6] A high concentration of biogenic amines can cause a multitude of negative health effects such as headaches, nausea, rashes and change in blood pressure. Often times, the biogenic amine concentrations are present in food matrices due to the presence of bacteria.[7] The bacteria will initialize the decarboxylation of amino acids to form the biogenic amines. This reaction is

shown in Equation 1. For this reason, biogenic amines will be present when certain bacteria are grown.[8] Therefore, there are two main processes that contribute to the biogenic amine presence in the human body these include biogenic amines that are naturally occurring and biogenic amines that are a result of bacterial flora. Additionally, biogenic amines can further undergo a process called amine oxidase, transforming the biogenic amines into aldehydes. With the introduction of water, the amine groups will be removed; therefore, resulting in the formation of aldehydes.

(1)

Amino Acid   Biogenic Amine   Aldehyde
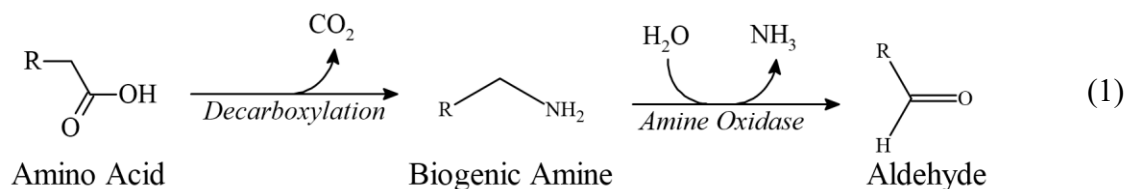
When death occurs, oxygen delivery inside the body ceases. Reactions in the body will naturally break down proteins into biogenic amines. Additionally, the cessation of oxygen delivery allows for anerobic bacteria to invade the body. The anerobic bacteria results in the production of biogenic amines. It is theorized that due to these two processes, the concentration of biogenic amines will increase as decomposition time increases. Our hypothesis is that the vitreous humor biogenic amine concentrations will change as a function of the time after death and this relationship can assist in more accurate estimation of PMI.

1.4 Biogenic Amines in Vitreous Humor
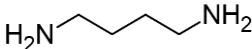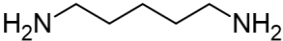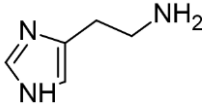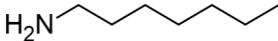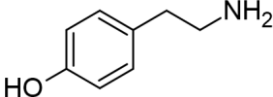
For this study, six biogenic amines will be analyzed in vitreous humor samples. These biogenic amines consist of putrescine, cadaverine, histamine, heptylamine, tyramine and methylamine. Each of these biogenic amines form naturally in the body as amino acids. Putrescine is produced by bacteria and fungi and is a result of ornithine decarboxylase. Cadaverine is synthesized from lysine decarboxylase enzyme activity. These two amines are commonly known to be present in decomposing tissues. Histamine plays more of a significant role in the body than the other biogenic amines that are involved in this study. Histamine consists of the histidine amino acid and is a result of histidine decarboxylase activity. In the body, histamine plays a part in balancing body temperature, stomach pH, and cerebral activity. Tyramine is one of the most common amines found in food. It is also synthesized from tyrosine decarboxylase. Inside the body, tyramine plays and important role in the increase of tears, saliva, and respiration.[9] Methylamine is a simple compound composed of only a methyl group and primary amine group; therefore, it has the lowest molecular weight of the biogenic amines in this study. It can be found naturally in the body due to the ingestion of choline and lecithin.[10] The structures of each of the biogenic amines used in this study are shown in Table 1. These amines were selected as potential candidates for PMI indicators.

1.5 Calibration and Internal Standardization

Internal standardization is a frequent practice when using chromatographic techniques, such as high-performance liquid chromatography (HPLC). In quantitative analysis methods, internal standards (IS) are often used to correct random and systematic errors.[11] Using an internal standard, accurate calibration of the instrument can be achieved by minimizing the matrix mismatch between the standard amine solutions and the matrix. Additionally, the effects of

instrument drift can be minimized by using a suitable internal standard. A good internal standard has similar chemical and structural properties to the analyte of interest; however, must not be present in the matrix. In this study, 1,7-diaminoheptane (Figure 2) was used as it is structurally and chemically similar to the targeted biogenic amines but is not present in vitreous humor.

Table 1. Biogenic amines and their related structures.

BIOGENIC AMINE	STRUCTURE
Methylamine	$-NH_2$
Putrescine	$H_2N\overset{\frown}{}NH_2$
Cadaverine	$H_2N\overset{\frown}{}NH_2$
Histamine	(imidazole)$-NH_2$
Heptylamine	$H_2N\overset{\frown}{}$
Tyramine	$HO-$(phenyl)$-NH_2$

$$H_2N\overset{\frown}{}NH_2$$
1,7-Diaminoheptane
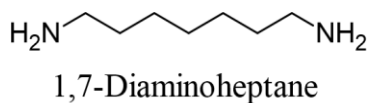
Figure 2. Structure of the 1,7-Diaminoheptane. Used as the internal standard.

The HPLC is commonly used in analytical chemistry using a high-pressure environment to separate, identify, and quantitate compounds that can be dissolved in liquid. Forensic

applications utilize HPLC due to its ability in identification and quantification of compounds in trace concentrations.[12]

1.6 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is one of the most widely used techniques for separation and analysis of compounds in mixtures. The HPLC instrument is composed of many parts that each play a crucial role in separation. The first part of HPLC is the solvent reservoir which holds the mobile phase. The mobile phase is a solvent that is pumped through the HPLC solvent delivery system. The flow rate of the mobile phase is dependent on the pressure in the solvent delivery system. A higher pressure will move the mobile phase quicker and a lower pressure will move the mobile phase slower. The samples are introduced into the continuous flow of the mobile phase using an injector, which will then be carried through the stationary phase. In the case of HPLC, the stationary phase is made of sorbent material in the form of particles that are packed into a cylindrical column. The sorbent material is selected based on the mixture of compounds that need to be separated. The separation of this mixture is based on the interactions that occur between the stationary phase and the compounds. As analytes are carried through the column by the mobile phase, they are separated and eluted through detectors. There are several types of detectors that are used to detect the compounds eluted from the column. Analytes that absorb visible or ultraviolet radiation can be detected by ultraviolet-visible spectrophotometry (UV/VIS). This detection method is used as a detection method in this thesis research. A more powerful, but destructive detection technique is mass spectrometry, which can be used to give qualitative and quantitative information. Detectors will produce electrical signals

based on the number of molecules passing through them. After passing through the detector the samples will then flow into waste.[12]

1.7 HPLC Separation Characteristics

When using HPLC, it is important that analytes are well-resolved. There are three primary characteristics of chemical compounds that contribute to HPLC separation. These include polarity, electrical charge, and molecular size.

Polarity is determined by the arrangement of a molecule's atoms and the bonds between them. The atom and bond interactions create specific molecular structure, activity, and physiochemical characteristics. These specific structures, called functional groups, determine whether a molecule is polar or non-polar. Since polar compounds are attracted to other polar compounds and non-polar compounds are attracted to other non-polar compounds, the characteristics of the mobile phases and stationary phase of HPLC are important for separation of solutes.[12]

There are two main modes of HPLC, reverse phase (RP) and normal phase (NP). The differences in these mechanisms are dependent on the polarity of the stationary and mobile phases. In RP-HPLC, the stationary phase in the column is nonpolar in nature while the mobile phase is a polar solvent. The analytes that have nonpolar functional groups will retain more in the column if they are non-polar in nature. Thus, having longer retention times. Additionally, the retention time can be increased by having a more polar mobile phase. Here, the analytes will interact, hence retained, more in the column and interact less with the mobile phase. The polarity of the analyte results in a specific retention time and can be altered depending on the mobile phase composition and the polarity of the stationary phase .[13]

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The stationary phase contains packing material that can range from various non-polar compounds when using RP-HPLC. Analytes that have similar polarity will retain on the column, whereas those that do not will elute off the column with the mobile phases.[12] In RP-HPLC the mobile phases typically feature a mixture aqueous and organic solvents. As the percentage of organic solvents are increased, the retention times of the analytes decrease. [14]

1.8 Mobile Phases

There are two types of elution methods, isocratic and gradient elution. Isocratic elution is a fixed mobile phase composition and works for most sample mixtures. The mobile composition does not change throughout the analysis time. However, if the sample is a complex mixture often a single mobile phase composition is not sufficient for successful separation due to having widely differing retention properties of the compounds in a mixture. This is known as the *general elution problem.* Therefore, most analytical methods employ gradient elution methods for mixtures with multiple compounds with different relative retention times.[15] Gradient elution is a change in the mobile phase during the analysis time. Typically increasing the amount of organic mobile phase will cause the mobile phase will become stronger eluents so that more nonpolar analytes can elute off the nonpolar column.[14] Alternatively, NP-HPLC utilizes a polar stationary phase and a nonpolar mobile phase. However, due to physical chemical properties such as the octanol-water partition coefficient (logP) and solubility in water, biogenic amines can be successfully retained and separated via RP-HPLC.[16]

1.9 Column Characteristics

To maximize selectivity, the column (stationary phase) and mobile phases must be carefully match. Picking the correct column for the analytes is a crucial step in chromatographic separation. Chemical properties including the functional groups attached or bonded to silica particles (column

packing materials) plays an important role in successful separation of compounds. The column packing material can be dependent on whether the analysis is conducted in RP or NP modes. For RP-HPLC, the most widely used method, non-polar packing material such as C18, C8 or phenyl bonded silica are commonly used as stationary phases. Alternatively, NP-HPLC will utilize polar packing materials such as silica. Factors that should be considered in the column are the particle size, pore size, packing material, internal diameter, and column length. Each of these properties can be separated into chemical or physical properties. The chemical properties allow selective separation and include pore size and packing material. The pore size is important for allowing molecules to flow through the column. Physical properties of the column include particle size, column length and diameter. For columns that have a smaller particle size, there is an increase in efficiency and backpressure. Depending on the particle size, the longer column length and narrower diameter will also increase efficiency; however, there will be longer run times, increased solvent usage, and increased backpressure. Shorter columns do minimize these variables but there will be a decrease in efficiency.[12]

1.10 UV/VIS Spectrophotometry

After the analytes are eluted off the column, the separated compounds move through the detector. When using HPLC for the separation of biogenic amines, common detection methods are UV/VIS, mass spectrometry, and fluorescence.

Detection of analytes using UV/VIS is the most common method of HPLC detection in use, as most analytes of interest absorb in the UV or visible region. When using UV/VIS, the concentration is returned as absorbance. The absorbance is determined by the fraction of light transmitted through the detector cell, which can be calculated using Beer's Law shown in Equation 2. Where A is the absorbance, I_o is the incident light intensity, I is the transmitted light intensity, ϵ

is the molar extinction coefficient, b is the path length of the cell in cm and c is the sample concentration in molarity.

$$A = \log\frac{I_o}{I} = \epsilon b c \qquad (2)$$

UV absorbance in the range detectable by HPLC occurs due to electron transitions from $\pi{\rightarrow}\pi^*$, $n{\rightarrow}\pi^*$, and $n{\rightarrow}\sigma^*$ molecular orbitals, in order of decreasing wavelength. This would mean that depending on the type of bonds an analyte has, the absorbance can differ. Typically, analytes with aromatic rings will absorb at 260 nm or less, analytes with one or more double bonds can be expected to have an absorbance of approximately 215 nm, and aliphatic compounds usually absorb at 205 nm. There are three types of UV/VIS detectors: fixed, variable, and photodiode array (PDA). The first models of HPLC were fixed detectors, meaning the wavelengths could not be moved beyond 254 nm. Alternatively, variable detectors can be tuned to different wavelengths according to the analyte. The lamp is directed through a slit to a diffraction grating. The diffraction grating will spread the light out into the UV/VIS wavelength spectrum. The grating is then adjusted to direct a single wavelength of light through a slit and flow cell to a photodiode, where the electrical signal is collected to generate a chromatogram. Additionally, a PDA is a variable detector; however, the light will pass through the flow cell before hitting the grating. This causes the light to spread the spectrum across the multiple detection elements of the photodiode array.[17] For this current study, an HPLC system is equipped with a variable wavelength UV/VIS detector.

1.11 Derivatizations

For the biogenic amines to be analyzed through UV/VIS, derivatization must be performed before introducing samples to HPLC. Smaller molecules such as biogenic amines can be tagged

by derivatizing agent that will significantly increase the UV/VIS absorption of these molecules. Derivatizing agents can help in various ways; however, for UV/VIS detection using derivatizations improve UV/VIS absorbance, stability and separation of the analytes.[18] For this study, dansyl chloride was chosen to be the derivatizing agent. Due to the high absorbance of UV light of dansyl chloride derivatives of amines, these amines can be detected at a very low concentration when HPLC is used with a UV/VIS detector. As shown in Equation 3, dansyl chloride can attach to each of the amine groups in the biogenic amine through nucleophilic substitution by the amine groups.[19] Depending on the concentration of dansyl chloride, the substitution can occur on both the primary and secondary amine groups[19].

Dansyl Chloride   Biogenic Amine   Derivatized Biogenic Amine

(3)
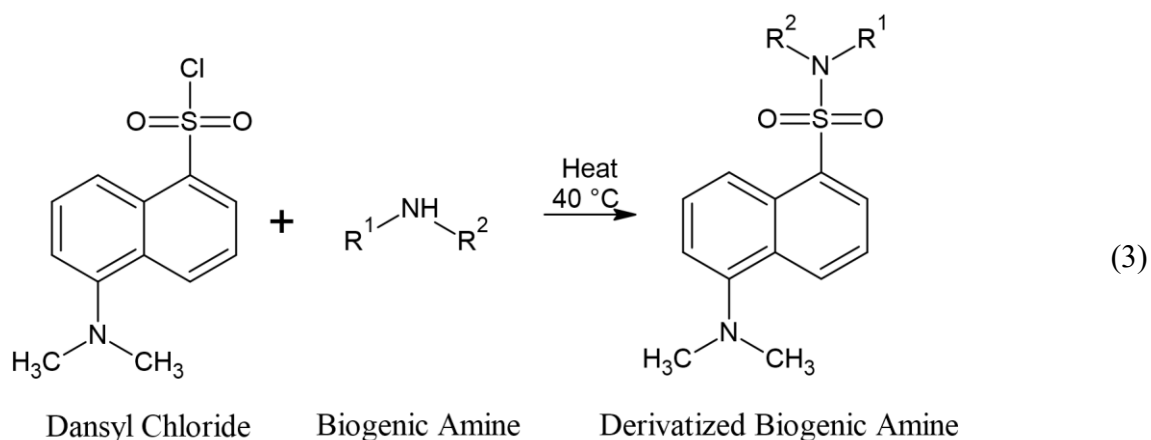
CHAPTER 2: METHODOLOGY

2.1 Materials

Biogenic amines used in this study were methylamine (Sigma Aldrich), putrescine (Thermo Scientific), cadaverine (Thermo Scientific), histamine (Alfa Aesar), heptylamine (Sigma Aldrich), and tyramine (Sigma Aldrich). A stock solution of 1000 ppm was made by diluting the appropriate amounts of amines in deionized water. Using 1,7 diaminoheptane (TCI) as the internal standard, a 1000 ppm stock solution was also made in deionized water. For derivatizations, 30 mg/mL of dansyl chloride (Thermo Scientific) was diluted using filtered HPLC grade acetone. 2 M sodium hydroxide was made in water by diluting a 10 M sodium hydroxide (Fisher) stock solution, and a saturated bicarbonate solution was made by diluting sodium bicarbonate (Fisher) in water. Additionally, 25% ammonium hydroxide (Fisher) was used in the derivatization reaction. The mobile phases used in the HPLC analysis include 0.1 M ammonium acetate (Fisher) dissolved in nanopore water and HPLC grade acetonitrile (Fisher).

2.2 Porcine Eyes

The experiments were conducted in a room temperature (20-22 °C) laboratory. Porcine eyes were collected from Neese's Sausage company located in Greensboro, NC. The eyes were picked up at the day of the dispatch and delivered directly to the lab.

2.3 Experimental Conditions and Sampling

Porcine eyes were stored in three different ways to investigate the effects of storing conditions. Although the conditions of which the eyes would experience when they are attached to the body cannot be reproduced, the goal of this experiment was to find the optimum conditions in

which the vitreous humor can be obtained for the longest period of time. First, a set of 50 porcine eyes were frozen for three days. After three days they were removed from the freezer and put into polyethylene bags to decompose at room temperature. Each day, three eyes were used to extract vitreous humor. Once the eyes were too decomposed to extract (approximately 9 days), vitreous humor collection was ceased. For the second experiment, 50 porcine eyes were collected and then immediately separated into polyethylene bags to decompose at room temperature. Again, for each day of decomposition three eyes were used to extract vitreous humor until collection was no longer effective (approximately 9 days). The last experiment conducted involved 60 eyes being placed in a polyethylene terephthalate box sealed with parafilm to desiccate at room temperature. Each day, three eyes were selected for vitreous humor extraction. The extraction continued until the vitreous humor could no longer be collected as a result of dehydration (approximately 7 days). For vitreous humor collection, the eyes were cut at the sclera to avoid cutting into lens. If the lens is cut, the vitreous humor can be contaminated with the aqueous humor. The vitreous humor samples were then kept frozen in centrifuge tubes until analysis.

2. 4 Derivatization

Once ready for analysis, the vitreous humor samples were removed from the freezer to reach room temperature. When equilibrium was achieved, the samples were centrifuged for 5 minutes at 4200 rounds per minute (RPM), then the supernatant was removed and derivatized for analysis. In a clean centrifuge tube, 800 µL of the vitreous humor supernatant is added, along with 200 µL of the internal standard, 200 µL of 2 M NaOH, 300 µL of saturated bicarbonate, and 2000 µL of the dansyl chloride solution (30 mg/mL). The solution was mixed using a vortex mixer for approximately 5 seconds. The solution was then heated using a solid state block heater for 45 minutes at 40 °C. Then, the solution was equilibrated to room temperature and 100 µL ammonium

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hydroxide was added, mixed, and left to sit for 30 minutes. After 30 minutes, the solution was centrifuged for 10 minutes at 4000 RPM. The supernatant is removed and filtered through a 0.22 μm filter and analyzed using HPLC.

2.5 Calibration

A series of known mixed biogenic amines solutions were created using concentrations of 250, 100, 50, 25, 10, 5.0, and 2.0 ppm. Each calibrator included methylamine, putrescine, cadaverine, histamine, heptylamine, and tyramine. These calibrators were derivatized using the procedure stated in section 2.3. The linear range for the calibration was found to be 2-250 ppm. Detector saturation was observed at higher concentrations while significant noise was observed at 1 ppm.

2.6 HPLC Analysis

An Agilent 1220LC equipped with a binary gradient pump, 100 position autosampler, and variable wavelength detector was used in this study. A Phenomenex Kinetex XB-C18 was used at 1 mL/min flow rate (see Table: 3). Two mobile phases were used with a gradient elution. Mobile phase A consists of 0.1 M ammonium acetate dissolved in nano pure water and mobile phase B was HPLC grade acetonitrile. The mobile phase gradient can be found in Table 4. A UV/VIS detector was used to detect derivatized amines.

CHAPTER 3: HPLC METHOD DEVELOPMENT

In order for HPLC analysis to be successful, each analyte must have baseline separation and individual peaks of the chromatogram should be separated from each other. Early phases of this biogenic amine study included determining the concentrations of biogenic amines including putrescine, cadaverine, histamine, heptylamine, and tyramine in soil under cadavers and in cadaver dog aids. The purpose of this was to determine the presence of biogenic amines in soil, then quantitatively compare the concentrations of the biogenic amines in the soil to the biogenic amine concentrations used in cadaver dog aids.

Soil was collected underneath decomposing cadavers at various stages of decomposition, then analyzed using what will be called the *Initial Method*. The Initial Method is the first set of HPLC parameters used to analyze the soil, cadaver dog training aids, and early vitreous humor samples. The parameters can be seen in Table 2, which expresses the column, flow rate, wavelength, mobile phases, gradient and injection volume used to analyze the analytes.

When initially analyzing the data, individual biogenic amine standards were made to determine the retention times using the parameters as shown in Table 2. These standards were then compared to the peaks from the cadaver dog aids and soil chromatograms. These resulted in the soil samples showing small concentrations of cadaverine and putrescine peaks. From that information, cadaverine and putrescine were the only biogenic amines that were being analyzed. Putrescine and cadaverine were the focus of the research for a majority of the analysis, until vitreous humor samples were introduced.

Table 2. HPLC parameters for the *Initial Method.*

Parameters	Condition		
Column	Avantor Prevail Select, 250 x 4.6 mm, 5 μm		
Flow rate	1 mL/min		
Wavelength	254 nm		
Injection volume	10 μL		
Mobile phases	0.1 M Ammoniums Acetate (A): Acetonitrile (B)		
Gradient	*Time*	*%A*	*%B*
	0.00	65	35
	5.00	55	45
	10.00	35	65
	17.05	20	80
	24	65	35
	30	65	35

Vitreous humor samples were analyzed using the same HPLC parameters as the soil and cadaver dog aid samples. Since there was a new matrix introduced, the five biogenic amines, putrescine, cadaverine, histamine, heptylamine, and tyramine, in addition to methylamine were used in a mixed amine sample to be compared to the vitreous humor samples to identify if there were any biogenic amines present. Histamine was added due to the possibility of having it in porcine tissues including vitreous humor.

The vitreous humor samples and mixed amine standards showed an obvious overlap between cadaverine and histamine which is shown in Figure 3. This was confirmed by injecting individual cadaverine and histamine standards at 1000 ppm. The overlapping of the biogenic amines is also shown in Figure 4. In order to fix the overlap between the two analytes they must be separated, which can be achieved through a few changes in the HPLC parameters.
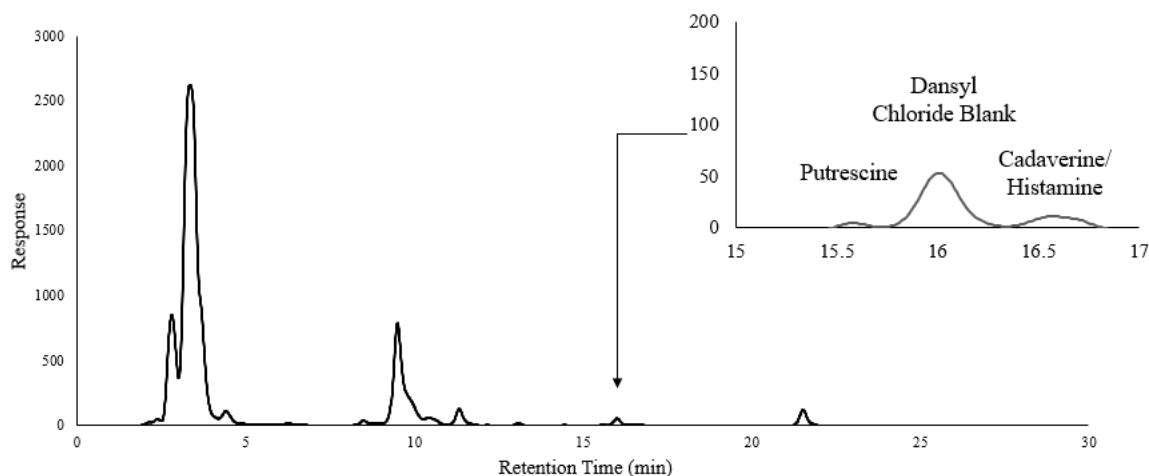
Figure 3. Chromatogram of vitreous humor collected on day 6 of decomposition collected using the *Initial Method*. Coelution of cadaverine and histamine is evident.
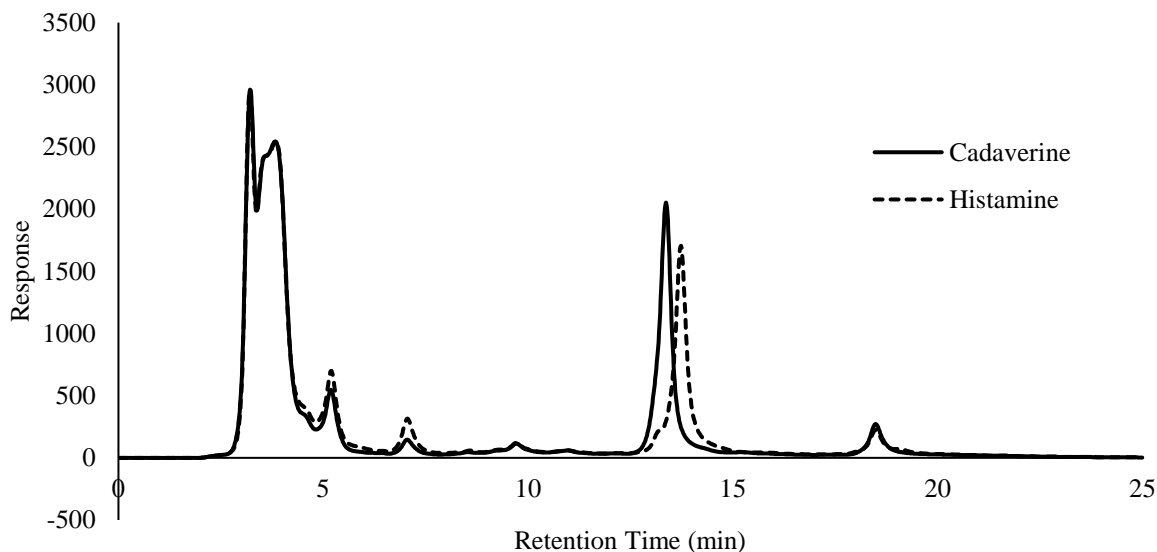
Figure 4. A chromatogram of a 1000 ppm standard of cadaverine and histamine overlayed to show the coelution of the biogenic amines using the *Initial Method*.

Separation can be affected by the concentration of dansyl chloride and the structure of the derivatized compounds. Since dansyl chloride has the ability to attach to both primary and secondary amine groups the physical and chemical properties of the biogenic amines can differ depending on the concentration of dansyl chloride. If dansyl chloride has a lower concentration, it may not attach to both amine groups, which will change the structure of the dansylated amine, ultimately changing the polarity. The changes in polarity can then have an effect on the retention times of the compound. However, this is not ideal since there will be inconsistencies in which amine group attaches to dansyl chloride. Having dansyl chloride in excess will ensure that each amine group in the biogenic amines attach to dansyl chloride. Additionally, the overall structure of the compounds has an effect on separation. The polarity can be affected by the linearity or nonlinearity of the structure, which in turn, affects the retention of such compounds in HPLC

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column. Compounds that are nonlinear can be bulky and depending on the pore size of the column, can have different retention times.

Separation of compounds using liquid chromatographic techniques can be achieved by optimizing multiple parameters. These include the mobile phase composition, stationary phase, and column characteristics including the column length, particle size, pore size. For the first step to achieve baseline separation between cadaverine and histamine, changing the mobile phase gradient first seemed like the best option. For each column discussed below, a series of isocratic elutions were made to study the retention behavior. The mobile phases that were used were an aqueous solvent of 0.1 M ammonium acetate and an organic solvent, acetonitrile. The mobile phase ratio is important for separation since the polarity determines the retention times of the analytes. If there is a higher ratio of the aqueous phase, the more polar analytes will elute faster, resulting in shorter retention times. For cadaverine and histamine, the polarity can be estimated by examining the chemical structures. Cadaverine is composed of a hydrocarbon chain consisting of five carbon atoms and two amine groups on both ends of the structure. Histamine is composed of a hydrocarbon chain with five carbon atoms with a single amine group on one side of the structure. It can then be assumed that histamine will be less polar than cadaverine due to the extra carbons in the chain and therefore will be eluted from the column later than cadaverine. Additionally, a gradient that contains a higher organic ratio may allow histamine to be eluted later than cadaverine and therefore allow the biogenic amines to separate.

The general workflow for the method development is as follows. First, a series of isocratic elutions were tested for each column. These were generally started from 30% of one mobile phase and 70% of the other. The mobile phase compositions were changed at about 10% increments to

observe the changes occur with the retention time and the separation of compounds. Then, a gradient elution was designed for each column to maximize the separation.

If adjusting the mobile phase gradient is not successful in baseline separation, as it was in this case, using a different column may be necessary. Different columns could possess unique properties, and this can benefit in separation depending on the analyte. For this study, each column is composed of a C-18 stationary phase. The first column used was the Avantor Prevail C18-Select and was the original column used in the soil and liquid cadaver aid research. This column has a C18 chain that will remain fully extended under highly aqueous conditions which allows for retention to be effective for highly polar compounds.[20]After injecting mixed bioamine samples with numerous gradient elution and isocratic elution, it was determined that the best course of action would be to change the column as there was no baseline separation. The chromatogram in Figure 5 yields the best separation of compounds as a result of the elution using the *Initial Method*.

Time (min)	A%	B%
0.00	65	35
5.00	55	45
10.00	35	65
17.05	20	80
24.00	65	35

Figure 5. Chromatogram of mixed amine standard solution at 1000 ppm using Avantor Prevail C18-Select column under the gradient elution parameters shown in this figure where A is 0.1 M ammonium acetate and B is acetonitrile.

Time (min)	A%	B%
0.00	40	60
3.00	25	45
6.00	25	65
10.00	95	80
15.00	95	35
20.00	40	60

Figure 6. Chromatogram of mixed amine standard solution at 1000 ppm using Thermo Fisher Scientific BDS Hypersil™ column under the gradient elution parameters shown in this figure where A is 0.1 M ammonium acetate and B is acetonitrile.

The next column used in the study was Waters Atlantis T3. This column is suggested for separation of polar and nonpolar compounds. Additionally, unlike the other columns used, this was the only column with a 3 μm particle size, which increased efficiency while also shortening the analysis time.[21] It once again underwent similar gradient elution methods to the previous columns to better understand where cadaverine and histamine will begin to separate. When analyzing the mixed biogenic amine samples with the Waters column, there were sharp peaks for each of the analytes; however, there was some peak tailing, and the biogenic amines did not have baseline separation as shown in Figure 7. The best way to resolve peak tailing was to change the pH of the mobile phases; however, it was decided that keeping the mobile phases at the original pH due to the successful separation of other amines under these conditions. The goal was to investigate all the available columns using these mobile phases to find the optimum conditions.

Time (min)	A%	B%
0.00	40	60
6.00	25	75
8.00	25	75
13.00	95	5
20.00	95	5
21.00	40	60
30.00	40	60

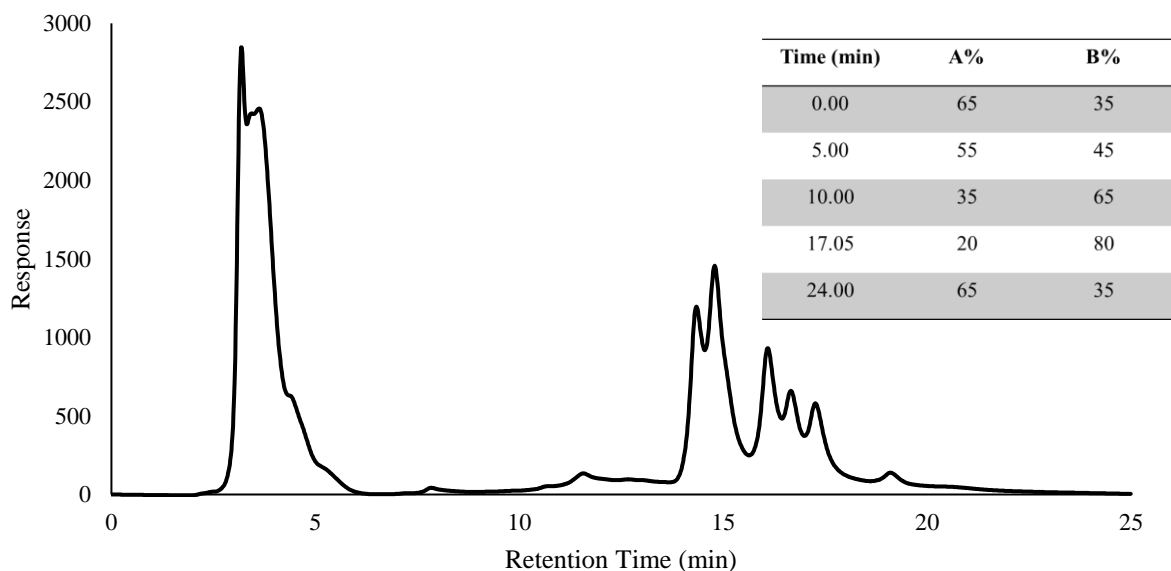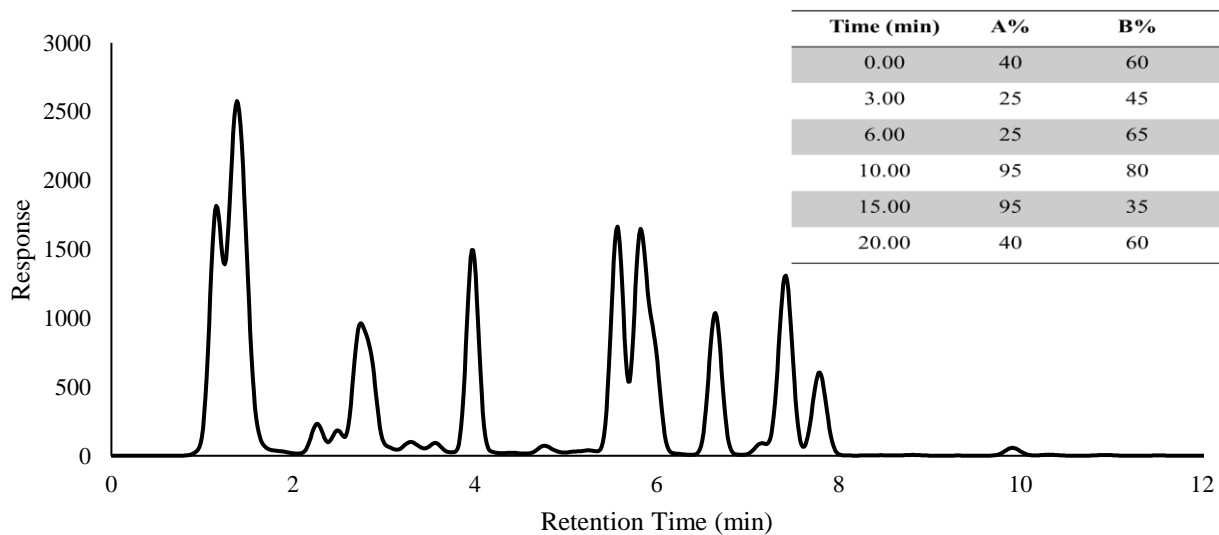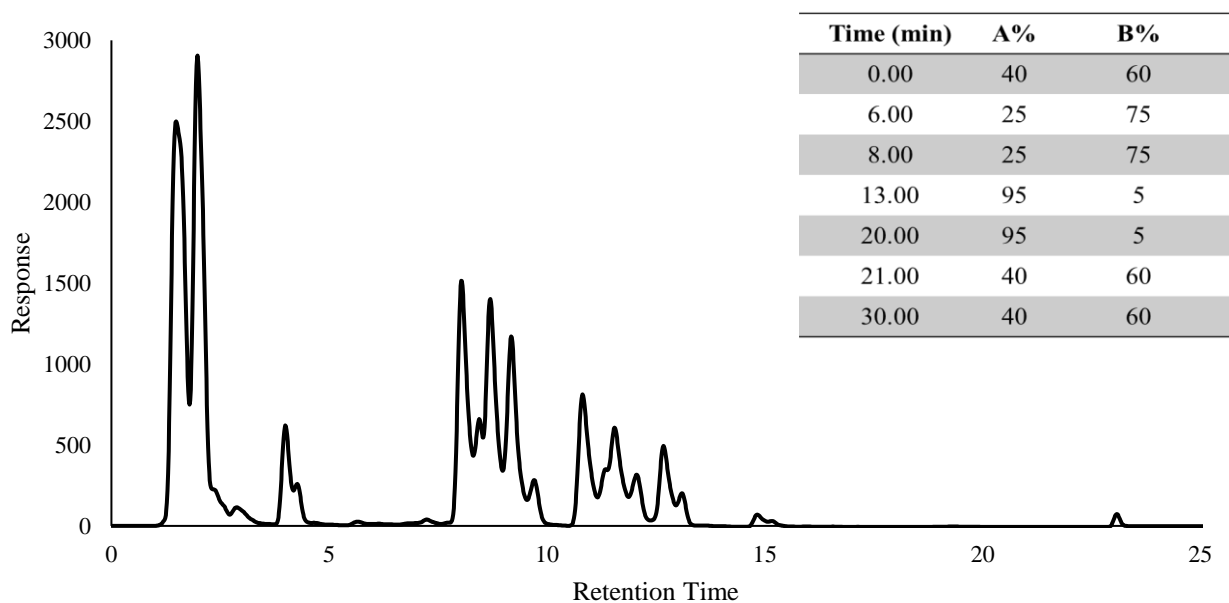**Figure 7.** Chromatogram of mixed amine standard solution at 1000 ppm using Waters T3 column under the gradient elution parameters shown this in figure where A is 0.1 M ammonium acetate and B is acetonitrile.

The final column used was a Phenomenex Kinetex XB-C18. This column is comprised of iso-butyl sidechains that yield increased hydrogen bonding that have an increased hydrophobic selectivity. This characteristic allows for improved peak shape for basic compounds and an increase in retention for acidic compounds. The issue with the original Avantor columns was the analyte peaks were broad, which was one of the reasons for the coelution. Since the biogenic amines are basic, the peak shapes should be improved, thus resulting in baseline separation of histamine and cadaverine. As shown in Figure 8, the peaks were resolved with the gradient elution shown.

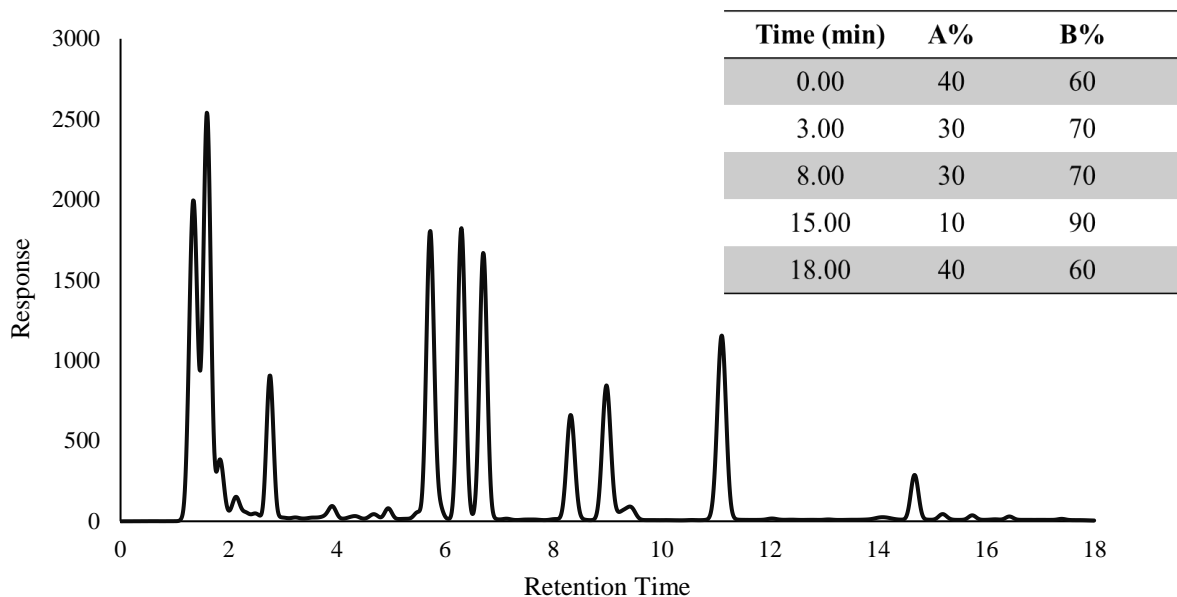| Time (min)	A%	B%
0.00	40	60
3.00	30	70
8.00	30	70
15.00	10	90
18.00	40	60

Figure 8. Chromatogram of mixed amine standard at 1000 ppm using Phenomenex Kinetex XB-C18 Column. Gradient elution parameters are shown in this figure where A is 0.1 M ammonium acetate and B is acetonitrile.

The Phenomenex Kinetex XB-C18 column was decided to be the best option to be used for the rest of the vitreous humor study due to the improvement in the peaks and the adequate separation of histamine and cadaverine. The gradient elution method was determined via isocratic elution. Isocratic elution was done for each of the columns except the Waters column. The elution of a ratio of 30 (A)/70 (B) for the Alltech, Phenomenex, and Thermo Scientific columns are shown below in Figure 9. This information allowed for the adjustments to be made to determine retention time of the analytes, to be able to build a gradient method that allows for the best separation possible. For the isocratic elution, a 500-ppm sample was used because often times in this study, higher concentrated analytes had broader peaks resulting in coelution. If the analytes are successfully separated using higher concentrations, it can be assumed that at lower concentrations there will not be any coelution.
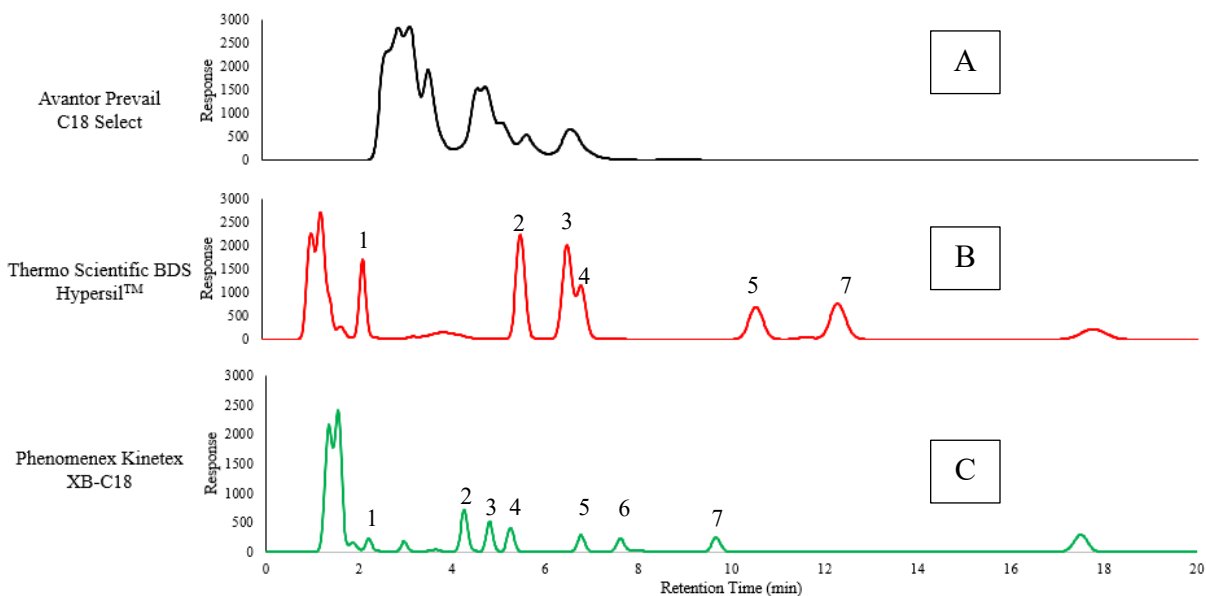
Figure 9. Comparison of chromatograms obtained by using the Avantor (A), Thermo Scientific (B) and Phenomenex (C) columns with isocratic elution at 30% is 0.1 M ammonium acetate and 70% is acetonitrile as the mobile phase. A 500 ppm mixed amine standard solution was injected. Elution order is (1) methylamine, (2) putrescine, (3) cadaverine, (4) histamine, (5), heptylamine, (6) internal standard, (7) tyramine.

Comparison of the chromatograms in Figure 9. show that the Phenomenex column has narrower peaks compared to other columns for amine standards. In total, four columns were used to determine what HPLC method best separated cadaverine and histamine. Each column and specifications can be seen in Table 3. Additionally, the new HPLC parameters can also be viewed in Table 4. The only parameters that changed were the mobile phase gradients and times and the column. All other parameters stayed the same including the mobile phases.

Table 3. Columns used for cadaverine and histamine separation with the column specifications.

Brand	Product Line	Stationary Phase	Length and Diameter (mm)	Particle Size (µm)	Pore Size (Å)	Part Number
Avantor [20]	Prevail	C18-Select	250 x 4.6	5	110	99301
Phenomenex [22]	Kinetex	XB-C18	150 x 4.6	5	100	00F-4605-E0
Thermo Scientific [23]	BDS Hypersil ™	C18	100 x 4.6	5	130	28105-104630
Waters [21]	Atlantis T3	C18	150 x 4.6	3	100	186003729

Table 4. Final Method parameters using the Phenomenex Kinetex XB-C18 column.

Parameters	Condition		
Column	Phenomenex Kinetex XB-C18		
Flow rate	1 mL/min		
Wavelength	254 nm		
Injection volume	10 µL		
Mobile phases	0.1 M Ammoniums Acetate (A): Acetonitrile (B)		
Gradient	*Time*	*%A*	*%B*
	0.00	40	60
	3.00	30	70
	8.00	30	70
	15.00	10	90
	18.00	40	60

CHAPTER 4: RESULTS AND DISCUSSION

The porcine eyes were decomposed in three different conditions to mimic different decomposition conditions.

4.1 Analysis of Vitreous Humor Collected from Frozen Eyes

In this study, there were three separate conditions that the vitreous humor samples underwent to determine if the conditions the eyes decompose in has an effect on the PMI. The first set of samples were collected on January 23rd, 2023. Before analysis they were frozen for three days.

4.1.1 Chromatograms

For each day of collection, three eyes were used for vitreous humor sampling then were analyzed using HPLC. Figure 10 is a representation of a chromatogram that was obtained from the frozen eye samples. The sample was collected on the 6th day of decomposition and is the second eye that was collected for that day. In the chromatogram there are peaks for methylamine (A), putrescine (B), cadaverine (C), histamine (D), and the internal standard (E). The remaining peaks are evident in the dansyl chloride blank as shown in Figure 11.
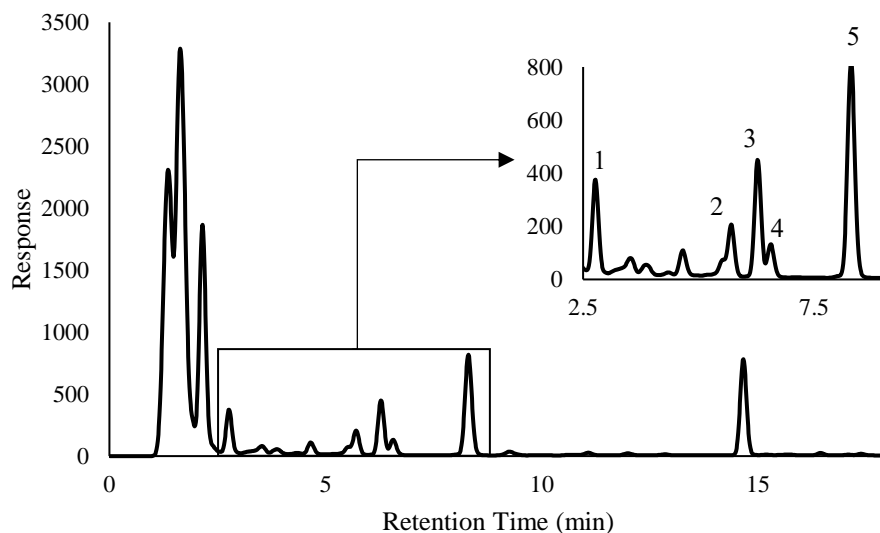
Figure 10. Chromatogram of frozen vitreous humor samples from day 6 of decomposition. Methylamine (1), putrescine (2), cadaverine (3), and histamine (4) are present in the sample. The internal standard is noted as peak 5.
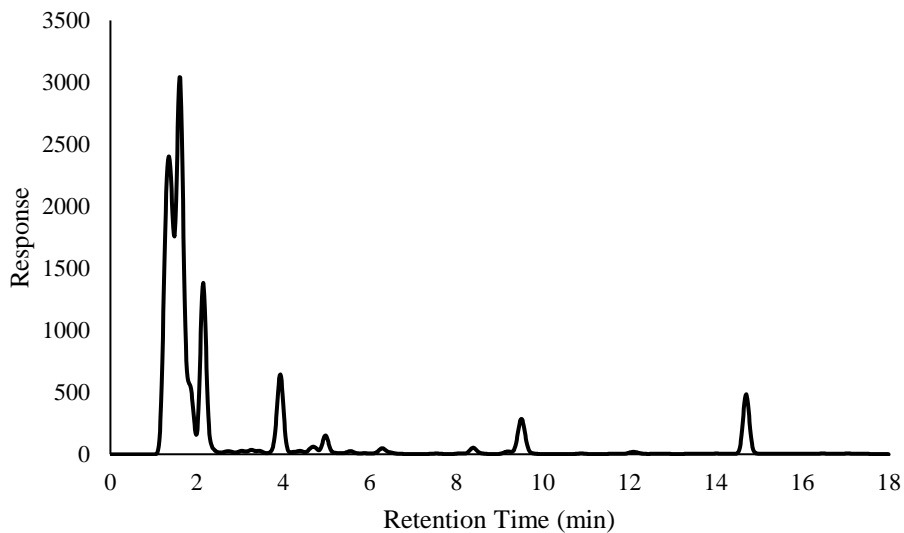

Figure 11. Chromatogram of a dansyl chloride blank. These peaks present in dansyl chloride are not representative of the biogenic amine standards or vitreous humor.

4.1.2 Calibration Curves

The calibration curves were created by using a set of known biogenic amine concentrations then determining the peak area ratio between the standards and the internal standard. These ratios were then plotted to create the calibration curve. Once the calibration curve was created, the line of regression can be determined and will be used to determine the biogenic amine concentrations, which are shown in the next section, **3.1.3**. Each of the calibration curves for the biogenic amines are represented below for methylamine (Figure 12), putrescine (Figure 13), cadaverine (Figure 14), and histamine (Figure 15).
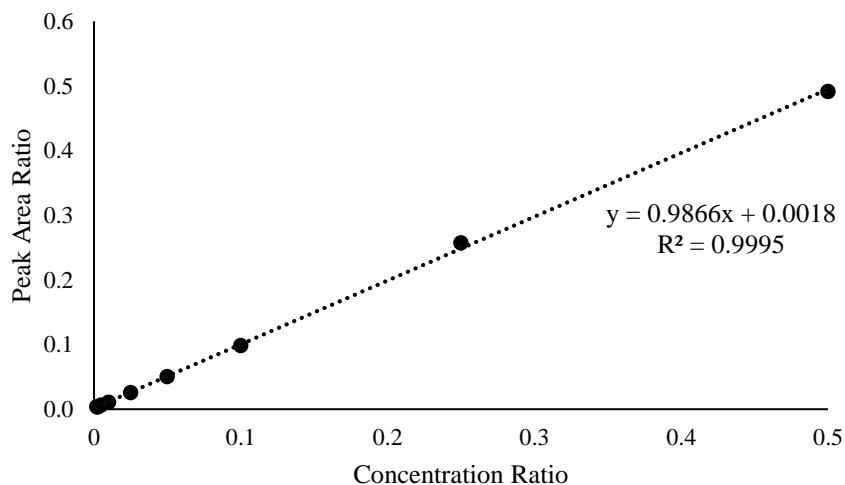

Figure 12. Calibration curve for methylamine standard used for frozen vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm. The liner regression equation was Peak Area Ratio = 0.9866(Concentration Ratio) + 0.0018. The coefficient of determination was 0.9995.

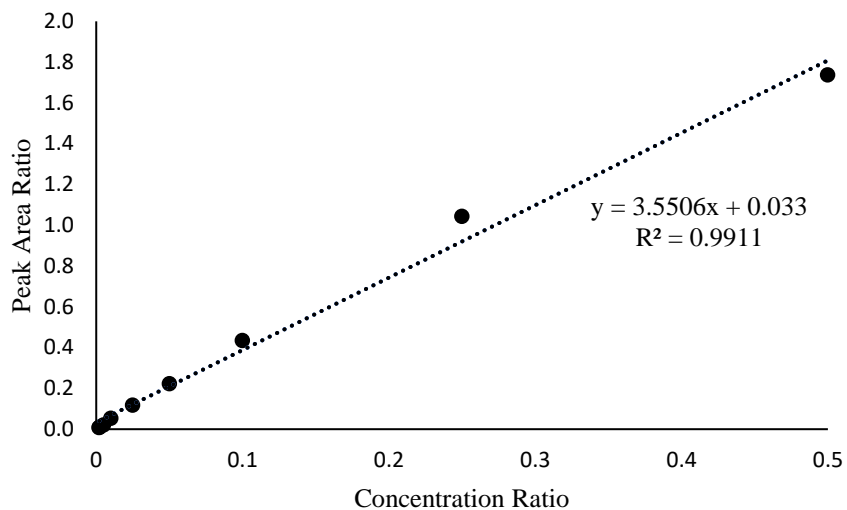**Figure 13**. Calibration curve for putrescine standard used for frozen vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.
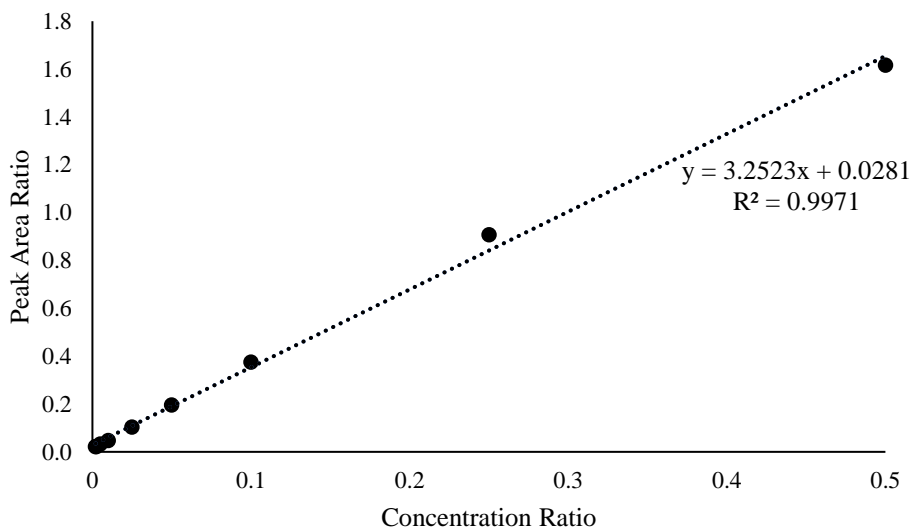

Figure 14. Calibration curve for cadaverine standard used for frozen vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.
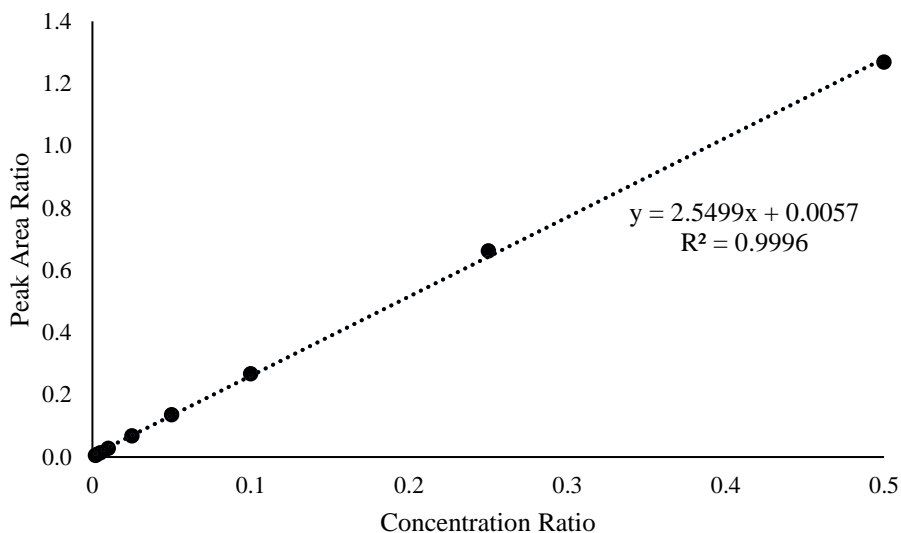
Figure 15. Calibration curve for histamine standard used for frozen vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.

4.1.3 Data Analysis

Methylamine, putrescine, cadaverine, and histamine was present during certain days of decomposition. The graphs of concentration of biogenic amines versus duration of decomposition are shown in Figures 16 to 19 for methylamine, putrescine, cadaverine, and histamine respectively. During this time, samples were collected each day until collection could no longer be done. Day 5 was not collected due to restricted access to the building.

Over the course of 8 days, the methylamine concentrations increased, then began to plateau. In order to understand further, analysis would need to be increased past an 8-day period; however, around this time the eyes become hard to work with and it is difficult to determine if there are any other substances in the eye such as the aqueous humor or blood from vessels in the eye (see Figure 16.).
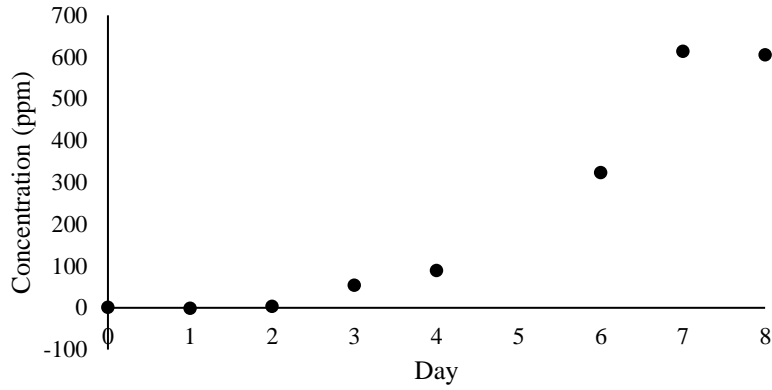
Figure 16. Concentration of methylamine in frozen vitreous humor over 8 days.

Putrescine was also present in the frozen vitreous humor samples. It is not present for day 0 or day 1 of decomposition. The increase in concentration is much more linear that the other biogenic amines that were found. This information could make determining the PMI of an unknown sample using putrescine easier than the other biogenic amines (see Figure 16.).
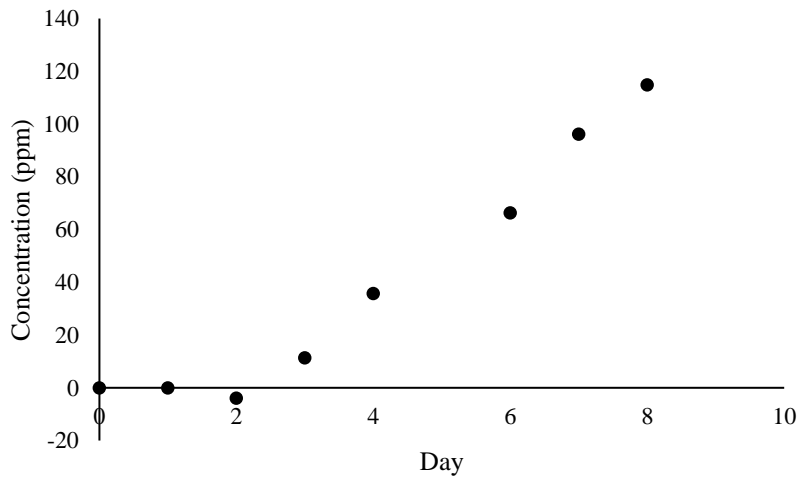
Figure 17. Concentration of putrescine in frozen vitreous humor over 8 days.

Cadaverine in the frozen samples increased as well over the course of 8 days; however, it forms at a slower rate compared to putrescine and began to increase at a higher rate between day 7 and 8. Analysis after day 8 would need to be performed in order to determine if the concentration plateaus, continues to increase, or decrease.
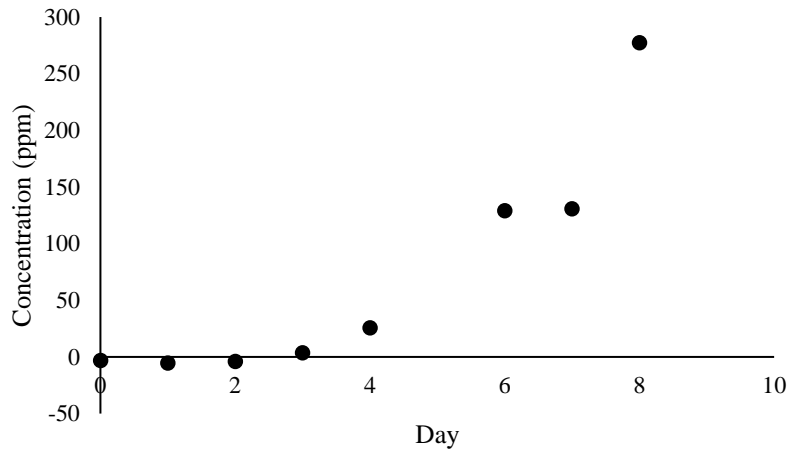
Figure 18. Concentration of cadaverine in frozen vitreous humor over 8 days.

Histamine increased in a similar pattern to cadaverine where the formation of the biogenic amine increased at a slow at steady rate, then between day 7 and 8, the increase is aggressive. As with the cadaverine analysis, the histamine analysis would need to be extended to more than 8 days to determine if the concentration plateaus, continues to increase, or begins to decrease.
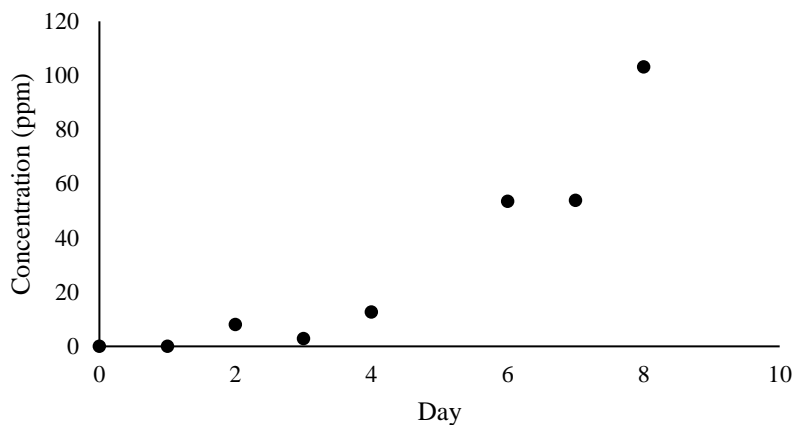
Figure 19. Concentration of histamine in frozen vitreous humor over 8 days.

4.2 Analysis of Vitreous Humor in Eyes Decomposed in Polyethylene Bags

The next set of eyes were analyzed without being frozen and were immediately separated into sets of three and put into a polyethylene bag. These eyes were collected on January 24th, 2024.

4.2.1 Chromatograms

An example of a chromatogram (Figure 20) from day 5 and the second eye from that sample day is shown below. Methylamine (A), putrescine (B), cadaverine (C), histamine (D) and the internal standard (E) can be seen in the chromatogram. Additionally, the dansyl chloride blank from this sample set is also shown in Figure 21. The dansyl chloride blank is the same blank that was also used for the vitreous humor samples that were decomposed in a box, since both sample sets were processed at the same time.

Figure 20. Chromatogram of bagged vitreous humor samples from day 5 of decomposition. Methylamine (1), putrescine (2), cadaverine (3), and histamine (4) are present in the sample. The internal standard is noted as peak 5.
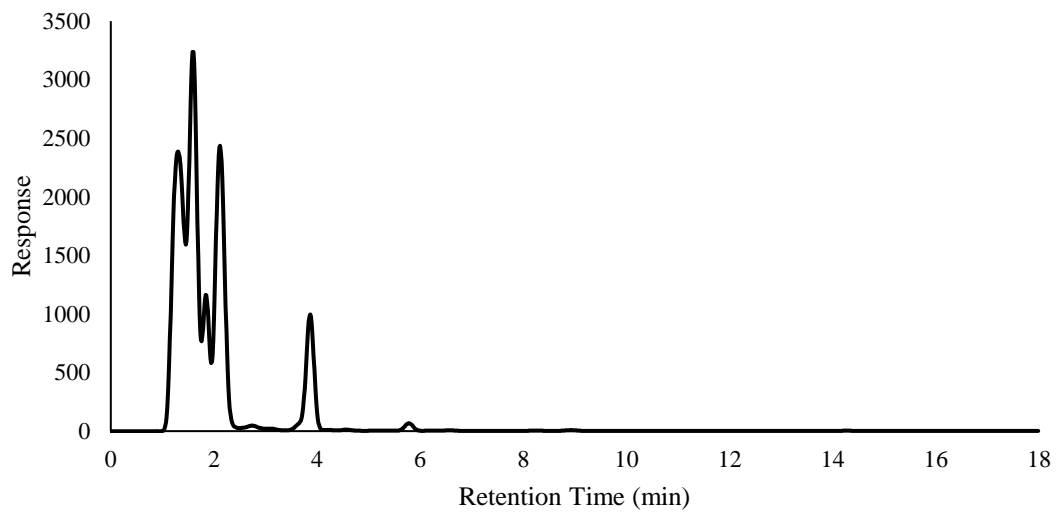
Figure 21. Chromatogram of a dansyl chloride blank. These peaks present in dansyl chloride are not representative of the biogenic amine standards or vitreous humor.
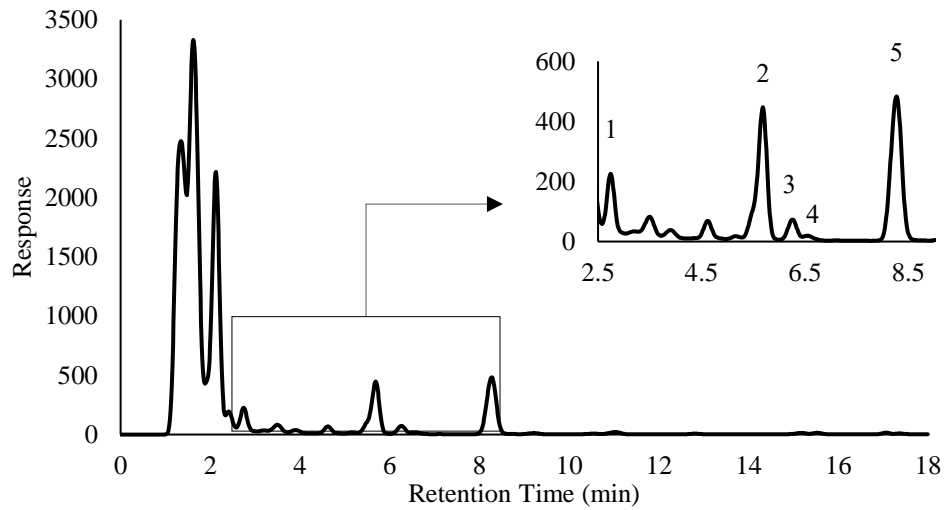
4.2.2 Calibration Curves

The calibration curves were used for eyes that were decomposed in both the bag and the box and are represented in Figures 22-25. Calculating the concentration was done in the same manner as the frozen eyes by obtaining the peak ratios between the standard peak area and the internal standard peak area, then plotting that with the concentration to get the linear regression line. The chromatograms of the samples were then used to get the peak areas of each biogenic amine then the peak area ratio of the internal standard was used to determine the concentration ratio from the linear regression line. Additionally, any vitreous humor samples that had a peak area above the peak area of the biogenic amine standard at 250 ppm was diluted to make sure that the sample analyzed is within the linier range of the calibration.
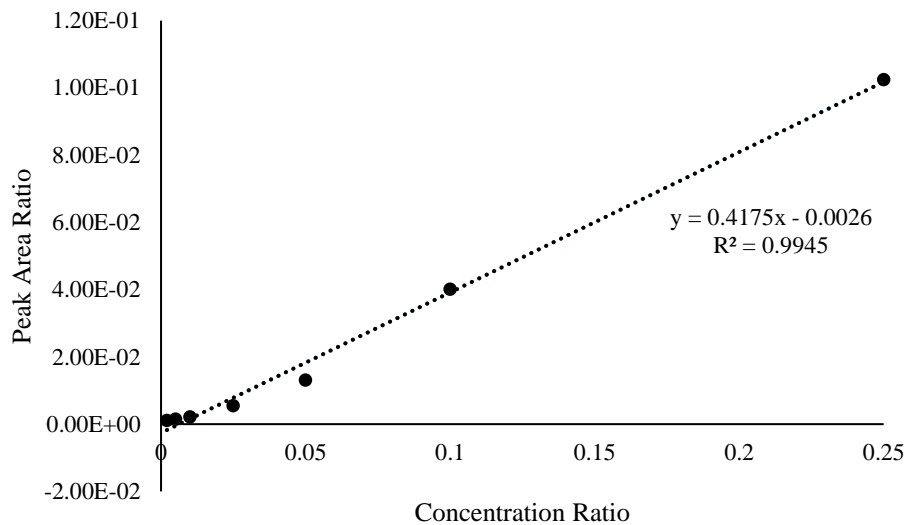
Figure 22. Calibration curve for methylamine standard used for bagged and boxed vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.
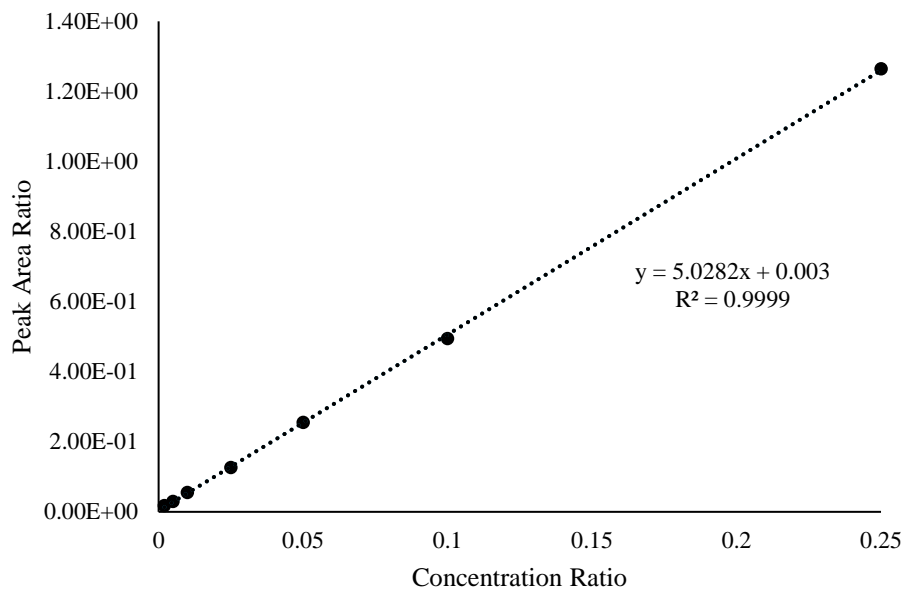
Figure 23. Calibration curve for putrescine standard used for bagged and boxed vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.
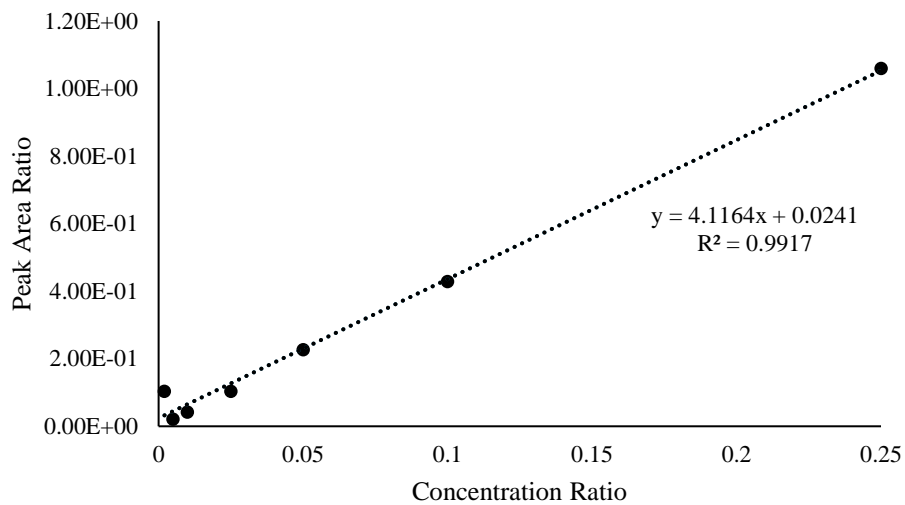
Figure 24. Calibration curve for cadaverine standard used for bagged and boxed vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.
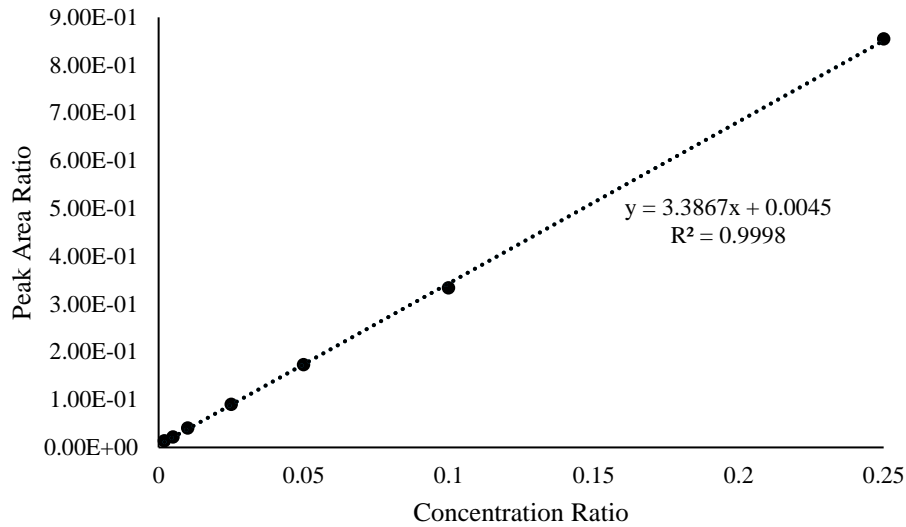
Figure 25. Calibration curve for histamine standard used for bagged and boxed vitreous humor samples. Calibrators ranged from 2 ppm to 250 ppm.

4.2.3 Data Analysis

This set also showed the formation of methylamine (Figure 26.), putrescine (Figure 27.), cadaverine (Figure 28.), and histamine (Figure 29.).

Methylamine is not present in the vitreous humor for the first days of decomposition. From day 3 to day 5 the concentration increases at a steady rate, then has a rapid increase in concentration from day 5 and day 6. During day 6 and day 7, the increase in methylamine concentration is aggressive, then plateaus on day 8.
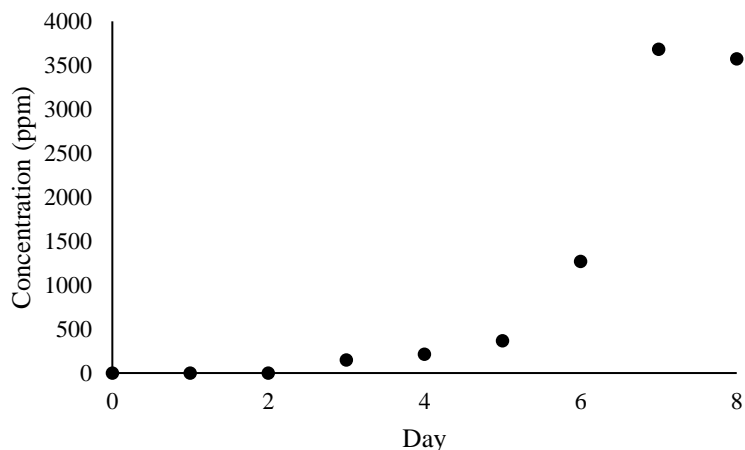
Figure 26. Concentration of methylamine in vitreous humor decomposed in a polyethylene bag over 8 days.

Putrescine was present in the vitreous humor samples during the entirety of the sampling process. The concentration increases at a rate similar to methylamine. For days 0 to 6 the increase in concentration is steady; however, during day 6 to 7 there is a rapid increase in putrescine concentration. On day 8, there is a slight increase in concentration, but may be indicative of a plateau.
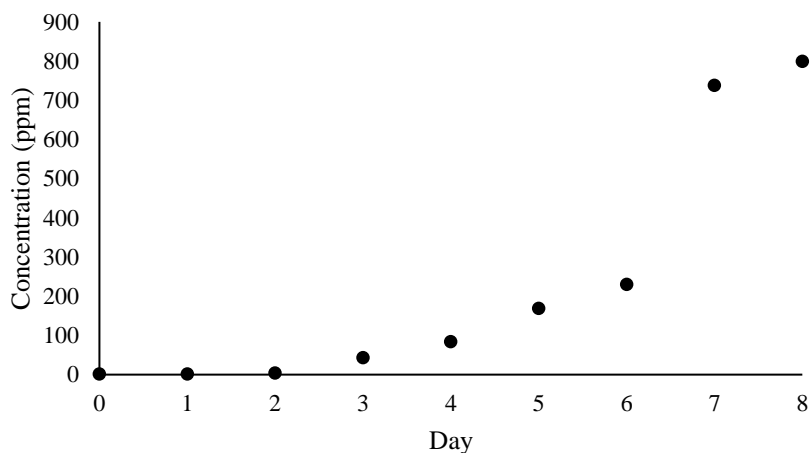
Figure 27. Concentration of putrescine in vitreous humor decomposed in a polyethylene bag over 8 days.

In the bagged samples, cadaverine follows a similar trend to methylamine and putrescine; however, the amine concentrations increase from day 0 to 3, then decreases from day 3 to 5, and then increases again from day 5 to 7. After day 7, the concentration begins to plateau.
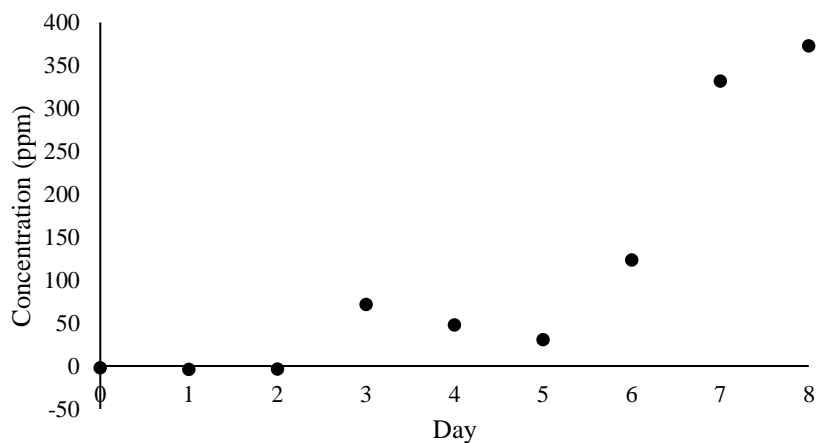
Figure 28. Concentration of cadaverine in vitreous humor decomposed in a polyethylene bag over 8 days.

The concentrations of histamine are low with the highest concentration being on day 5 with 4.78 ppm. In day 0 to 2 there is minimal amine formation, but day 3 to 5, there is an increase in concentration. After day 5, histamine is no longer present in the samples.
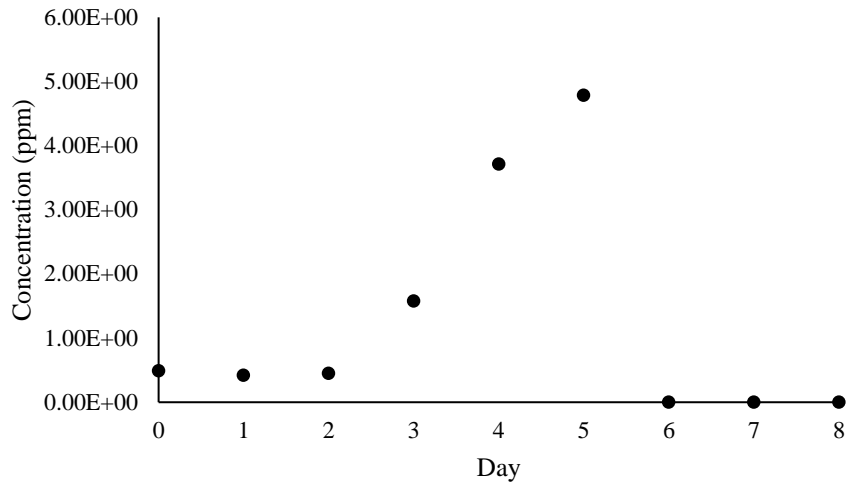
Figure 29. Concentration of histamine in vitreous humor decomposed in a polyethylene bag over 8 days.

4.3 Analysis of Vitreous Humor from Eyes Decomposed in a Polyethylene Terephthalate Box

The last set of eyes was a set of 60 that were placed in a clear polyethylene terephthalate container. The eyes were placed approximately 2 inches apart. Each day, eyes were used to sample the vitreous humor. This continued until there the vitreous humor could not be collected. On the sixth day of vitreous humor collection, only 200 µL of vitreous humor was retrieved and therefore was omitted from the data analysis.

4.3.1 Chromatogram

Figure 30. shows a chromatogram of the vitreous humor samples on day 5 of decomposition in a polyethylene terephthalate box.
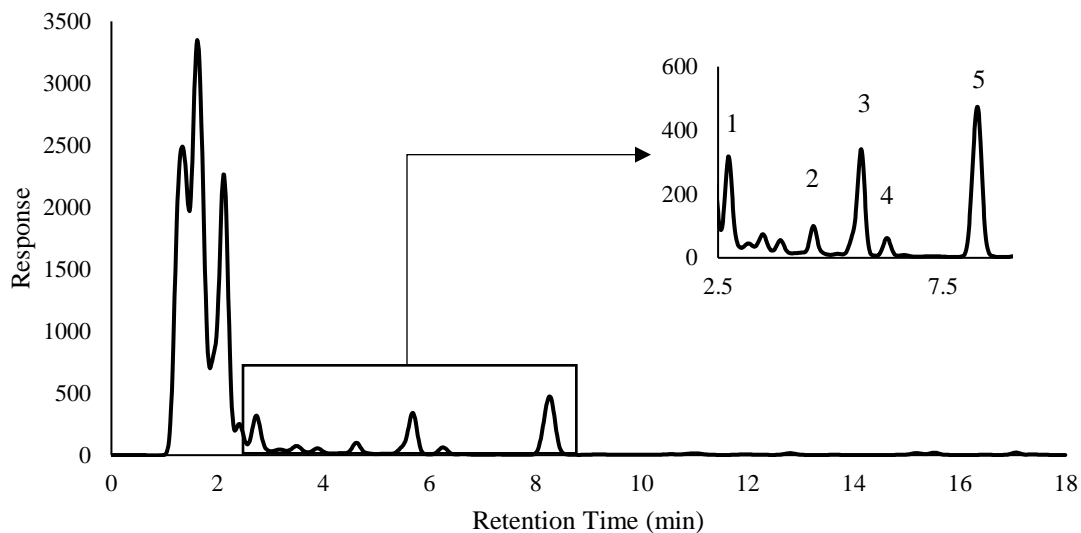
Figure 30. Chromatogram of the boxed vitreous humor samples from day 5 of decomposition. Methylamine (1), putrescine (2), cadaverine (3), and histamine (4) are present in the sample. The internal standard is noted as peak 5.

4.3.2 Data Analysis

Methylamine was already present in the vitreous humor. The concentration increases on day 3 then plateaus. From day 4 to 5, the concentration of methylamine begins to increase again. This is shown in Figure 31. It can be assumed that the concentration will continue to increase after the fifth day.
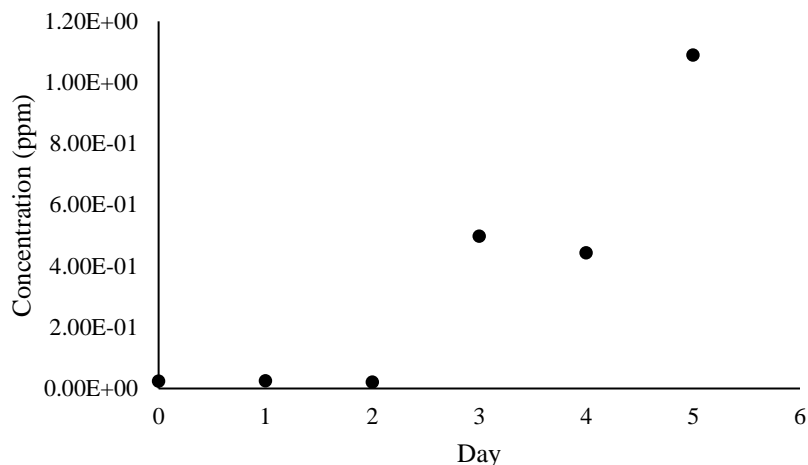
Figure 31. Concentration of methylamine in vitreous humor decomposed in a polyethylene terephthalate box over 6 days.

Putrescine concentrations are shown in Figure 32. Putrescine is present in the vitreous humor at low concentrations from the first day of sampling, then increases until day 5. Analysis for day 6 would be beneficial to determine if putrescine plateaus after day 5 then decreases between day 6 and 7 or if the amine concentrations are quenched after day 5.
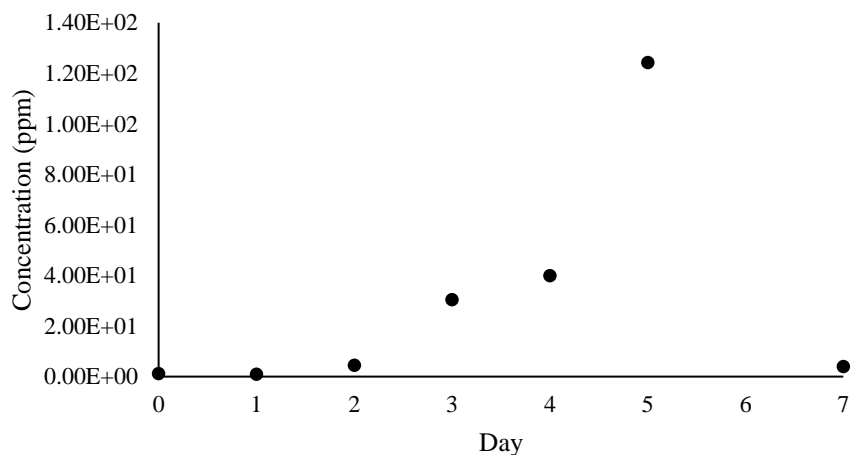
Figure 32. Concentration of putrescine in vitreous humor decomposed in a polyethylene terephthalate box over 6 days.

In the case of cadaverine, there is no pattern that can be determined when using a box for the decomposition environment, as shown in Figure 33. Cadaverine present some days such as day 3, 4, and 5; however, it does not follow any predictable pattern and therefore can be assumed that there is no cadaverine formation that is apparent and cannot be used to determine the PMI.
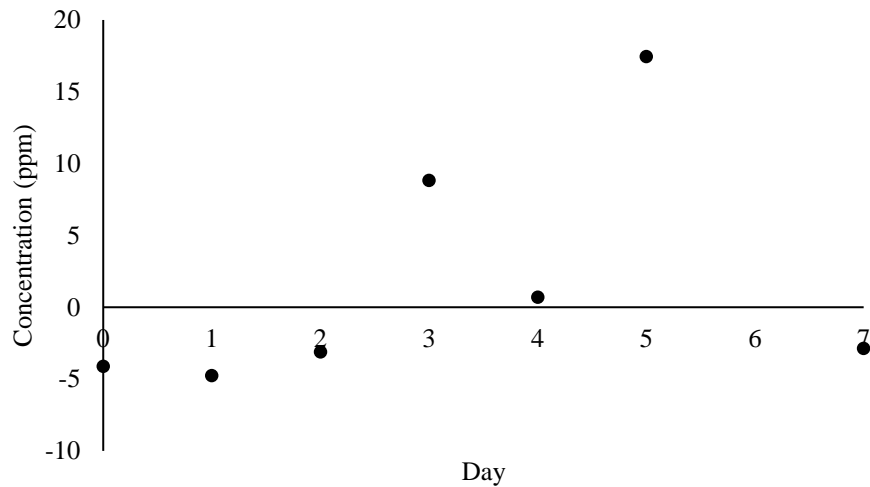
Figure 33. Concentration of cadaverine in vitreous humor decomposed in a polyethylene terephthalate box over 6 days.

Histamine maintained a consistent concentration throughout the collection period. Although histamine is present, there is no formation from day to day and therefore cannot be used to predict the PMI, which is represented Figure 34.
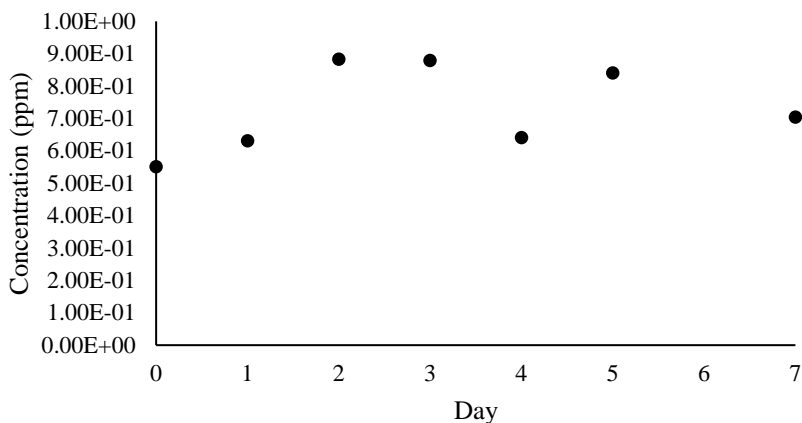
Figure 34. Concentration of methylamine in vitreous humor decomposed in a polyethylene terephthalate box over 6 days.

4.4 Discussion of Biogenic Amine Concentrations in Vitreous Humor

4.4.1 Methylamine

The methylamine concentration in the frozen eye sample set increases slowly then has an aggressive increase, then plateaus. This is also what occurs in the sample set with the bagged eyes; however, there is a slight decrease on day 8 which may indicate other reactions occurring in the vitreous humor. As the amino acids in the body start to decarboxylate, the concentration of methylamine increases. The plateau can be due to the there being no more of the amino acids being decarboxylated. In order to see what happens after the plateau, analysis would need to be done past 8 days; however, the vitreous humor collection became difficult because the tissues in the eye began to deteriorate. For this reason, the eyes were then put into a box to try to minimize any excess fluid that would deteriorate the eye. This method had a lower concentration of methylamine than the frozen and bag method but does still increase over time. Overall, using either the frozen or bag sample to determine the methylamine concentration for PMI could be beneficial. In

instances where the vitreous humor cannot be immediately processed, freezing the eyes will still follow a similar trend.

4.4.2 Putrescine

The rate putrescine increases in the frozen eye sample is at a constant rate. Although it is not present in the first few days of collection, the concentration increases rapidly after that. The importance of this relationship is that it is the only biogenic amine in this study that does not plateau. Once putrescine forms from an amino acid, it can form into spermidine or spermine, then back to putrescine. This could be why there is no plateau during the duration of the sampling period. This could be useful in determining the PMI if spermine and spermidine are also being formed, those concentrations could be considered as well. Another explanation is that putrescine is being formed at a slower rate and the plateau will occur after the 8-day period. For the bagged eyes, putrescine concentrations increase, then plateau which can indicate that the original amino acids cannot decarboxylate into putrescine and has reached the maximum concentration. After the plateau, putrescine can undergo amine oxidase, ultimately forming an aldehyde. For the boxed eyes, on day 7 the formation of putrescine is quenched. This can be due to the eyes drying out due to a lack of moisture in the box. For determination of PMI, the frozen eyes and the bagged eye would be beneficial. Once again, the concentration of putrescine in the bag is higher than the eyes that were frozen, but both have a predictable increase in concentration.

4.4.3 Cadaverine

The formation of cadaverine in the frozen samples slowly increased then plateaued, then increased again. The formation of cadaverine in frozen eyes follows a similar kinetics compared to bagged samples. It is difficult to determine if the bagged samples begin to plateau after the 8-day period, and further analysis will need to be done to investigate the change in concentrations.

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In the boxed samples, the concentrations fluctuate and do not follow a predictable pattern; therefore, would not be a valuable indication for determining the PMI.

4.4.4 Histamine

Histamine follows a similar pattern to cadaverine in frozen samples, where the formation begins to slowly, followed by the increase of the concentration. Then the concentration plateau followed by a rapid increase. The concentration of histamine in the frozen samples is comparatively higher than the other sample sets, which shows a different behavior compared toother biogenic amines. In the bagged sample set, histamine follows the 2nd order of kinetics as it increases slowly then has a rapid increase in concentration. The lower concentration in the bagged samples could be due to histamine bonding to other compounds in the vitreous humor or metabolized by N-methyltransferase which would have been introduced from other components in the eye.[24] Similar to cadaverine, the histamine concentration is low and has an unpredictable pattern, so would be difficult to use for PMI estimation.

4.4.5 Effects of Different Environmental Conditions

Each of the conditions that the eyes were subjected to had problems that could have inhibited the formation of biogenic amines. We hypothesized that freezing the eyes could destroy the cellular structure of eyes their delicate nature and inhibit the microbes present on the eyes. Biogenic amines can be produced by microbial flora and if microbial growth is inhibited, biogenic amine formation will also be diminished. If the cellular structure is destroyed, certain enzymes, such as N-methyltransferase, that inhibit biogenic amine formation can be introduced into the vitreous humor from the blood vessels or from the aqueous humor. This ultimately can have an impact on the overall estimation of the PMI. The issue of the destruction of the cellular structure was also a concern when decomposing the eyes in polyurethane bags. High moistures content

environment produced by the decomposing eyes and the high pressure caused by the bag walls will result in destruction of the cellular structures, thus leading to the introduction of enzymes and other biogenic amines that would not be a part of the vitreous humor. Comparison of data from the frozen and the bagged eyes suggest that this may have contributed to the behavior observed with the change in histamine concentration. It was presumed that the biogenic amine concentrations would follow a similar trend since eventually both experiments resulted in the eyes being decomposed in a bag. The concentration over time is very similar between the two environments; however, the bagged eyes have a significantly increase in biogenic amine concentration. The lower concentration of the frozen eyes can be indicative of the inhibition of microbial growth. Histamine concentrations, however, were significantly lower in the bagged eye experiment. It can be assumed that this was caused by the contamination of vitreous humor by the surrounding tissues and fluids in the eye. Enzymes such as N-methyltransferase can be present in the outer tissues and blood vessels and upon reaching into the vitreous humor they can inhibit the formation of histamine. The boxed eyes were in a completely different decomposition environment than the other two experiments. Due to the eyes becoming too soggy to extract the vitreous humor, the box was meant to maintain an environment that allowed the eyes to decompose naturally without the interference of other fluids in the eye. However, during decomposition the eyes became dehydrated. Dehydration can be attributed to the lower concentration of biogenic amines in the boxed eyes. Due to the desiccation of the eyes, the collection of vitreous humor ceased on day 7.

For many of the biogenic amines, the formation began to plateau towards the last few days of sampling. This could be attributed to all the amino acids that the biogenic amine is decarboxylated from is completely used up. This would result in the cessation of biogenic amine formation. Additionally, the biogenic amines can undergo amine oxidase which is the introduction

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to water which can cause the removal of the amine. This would create aldehydes and will cause the concentration of the biogenic amine to decrease. To account for that, further research can be done on the aldehydes present and a ratio between the biogenic amine formation and aldehyde can give an accurate representation of the PMI.

4.4.6 PMI Estimation

Finding a pattern in the formation of the biogenic amines was important in the contribution to the estimation of PMI. By analyzing multiple biogenic amines in different decomposing environments, the likelihood of finding a method that can be used to successfully estimate PMI increases. In order to completely understand if any pattern is present, more sample sets from the same decomposition environment would need to be analyzed. Understanding the environment that the biogenic amines form best in is useful when analyzing samples in a controlled environment; however, if this method is used in forensics applications, the environment during decomposition can vary; therefore, it is important to understand the behavior of the biogenic amine formation. Using the data obtained from this research, using either frozen or bagged eye samples have patterns that can be used to estimate the PMI. Additionally, methylamine and putrescine would be the biogenic amines that would give the best estimation of PMI. Both have significant formation increases throughout the duration of decomposition.

CHAPTER 5: SOIL AND LIQUID CADAVER DOG AIDS

Dogs are frequently used in a variety of police investigations. Due to their ease of trainability and sensitive nose, dogs have the ability to track down scents of people, explosives, drugs, even cadavers. Cadaver dogs are trained to recognize and react to specific scents in cadavers. Due to ethical issues in many countries, training must use pseudo-scents such as liquid cadaver dog aids, sometimes known as 'pseudo scents', that mimic the smell expressed by cadavers. Although cadaver dogs are successful in identifying cadavers, there is limited research done to establish if there is any relationship between the stage of decomposition a cadaver is in and the effectiveness of the liquid cadaver dog aids.[25] To determine the sensitivity cadaver dogs have to the scents expressed in cadavers, the concentration of biogenic amines in both cadaver grave soil and liquid cadaver dog aids can be compared. Grave soil can be a good source of experimentation, due to the soil collecting any decomposition fluids from the cadaver. Identifying and quantifying the biogenic amines present in cadaver dog aids and cadaver soil can indicate the accuracy pseudo-scents have to cadavers decomposing in different stages. This data could also be beneficial in estimating the PMI. By determining the concentration of biogenic amines at different stages of decomposition as a function of time, an additional correlation between the chemical composition and the PMI can be developed.

5.1 Soil Analysis

The initial goal of this research was to investigate the use of amine concentrations in grave soil to estimate the PMI. Using the *Initial Method*, it was found that putrescine, cadaverine and/or histamine were present in grave soil. The soil samples were collected from the Forensic Osteology

Research Station (FOREST) at Western Carolina University. A sample of 5 g of soil were collected from between the leg of bodies that are decomposing at the surface enclosure of the facility. These soil samples were derivatized and analyzed following the same procedure used for virtuous humor. The presence of histamine was not confirmed due the method used for the analysis of soil samples. Below, in Figure 35., is a chromatogram from a soil sample collected in April 2021. Preliminary data from the *Initial Method* did not show any specific patterns for the formation of the biogenic amines with PMI. Due to this reason, the focus of this research changed to the analysis of biogenic amines in vitreous humor. Using an internal standard calibration, putrescine cadaverine and/or histamine was determined by using the derivatization method used stated in methodology chapter of this thesis. Chromatogram of a soil sample collected April 2021 using the *Initial method*. Is shown in Figure 35. Peaks for putrescine are present, but there may be a coelution of cadaverine and histamine. The putrescine concentration was 264 ppm while cadaverine and/or histamine concentration was found to be 308 ppm. The analysis of multiple soil samples including controlled samples collected from a place outside the FOREST and the results showed no correlation between the stage of decomposition or PMI. Therefore, the analysis of soil was discontinued.
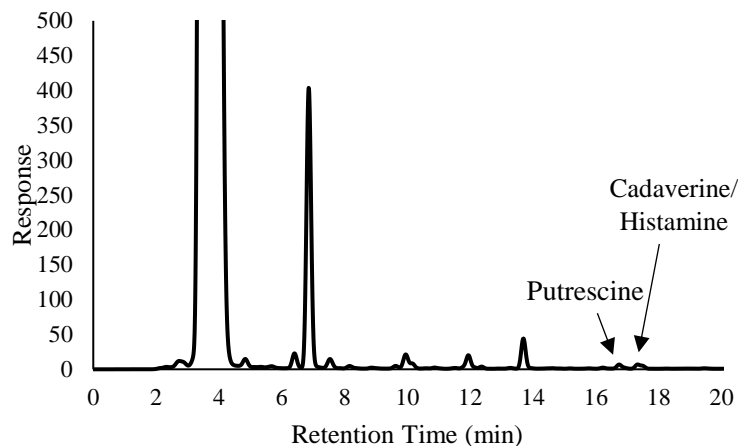
Figure 35. Chromatogram of a soil sample collected April 2021 using the Initial method. Peaks for putrescine are present, but there is coelution of cadaverine and histamine.

5.2 Cadaver Dog Training Aids

Similar to the soil analysis, the *Initial Method* was used to analyze cadaver dog training aids. Figure 36. represents data from liquid cadaver dog aid Sample 3929. These samples were received as liquids in ampules, and they were derivatized using the same procedure that was used for the vitreous humor samples. The chromatogram shows peaks for both putrescine and cadaverine. Sample 4304 is the chromatogram in Figure 37. where putrescine (A), cadaverine (B) and histamine (C) are present. Even though there are obvious peak present for the biogenic amines, they cannot be used in this research since the *Initial Method* did not account for the separation of histamine from cadaverine. Originally, a calibration curve of putrescine and cadaverine was created using the *Initial Method*. Sample 3929 was calculated to have a concentration of 6119 ppm putrescine and 3629 ppm cadaverine. Sample 4304 calculated a putrescine peak to be consistent with 56 ppm and a cadaverine peak calculated to be 35 ppm using the same calibration curve.

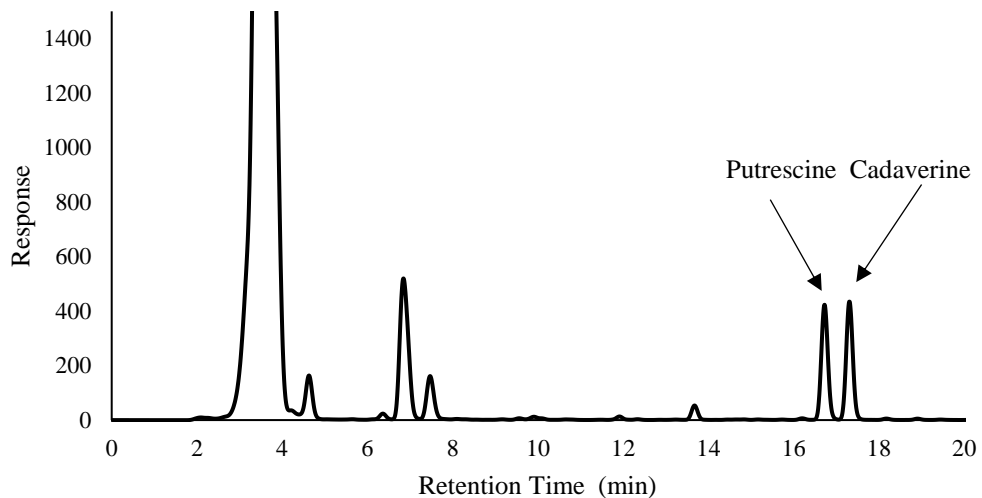**Figure 36**. Chromatogram of liquid cadaver dog aid Sample 3929 using the Initial method. There are putrescine and cadaverine peaks, but there may also be coelution with cadaverine and histamine.
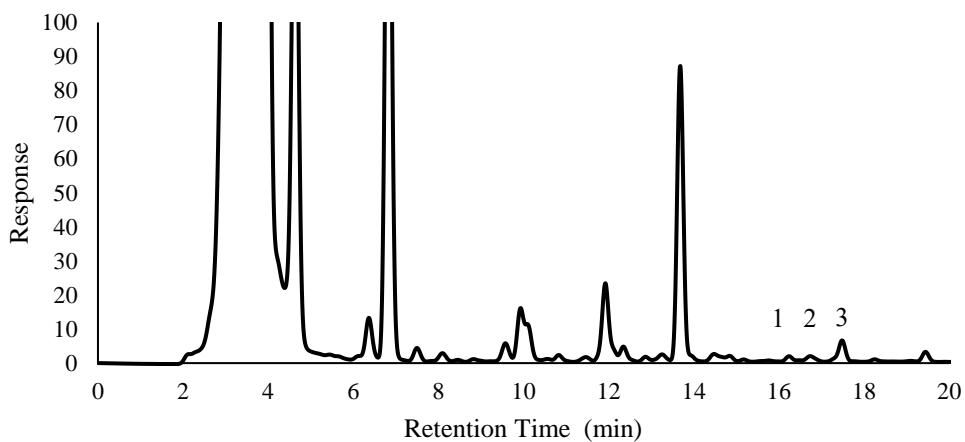

Figure 37. Chromatogram of liquid cadaver dog aid Sample 4304 using the Initial Method. There are putrescine (1), cadaverine (2), and histamine (3) peaks, but there may also be coelution with cadaverine and histamine.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

In criminal investigations, PMI is determined by employing various methods of analysis including the skeletal decomposition stage, the stage of death and potassium concentration in the vitreous humor. Often, skeletal decomposition related methods were used to determine longer PMI due to the use of physical changes occur during the decomposition. The other methods including stages of death such as liver mortis and rigor mortis, are subjective since they utilize physical properties of the cadavers, and they can be subjective of the analyst's opinion. Additionally, these methods are not suitable determining the PMI of a recently deceased person. Furthermore, the only method currently used in forensic labs that are supported by chemical evidence is the potassium concentration in the vitreous humor. As decomposition progresses, the PMI increases; thus, needing to employ a multifaceted approach to improve PMI accuracy. In conjunction with using the stage of death and the potassium content in the vitreous humor can be useful by extending the analysis up to a 5-day period. Therefore, the introduction of a new method could benefit the accurate estimation of PMI. Integrating biogenic amine concentrations into the PMI estimation can enhance the precision in determining the time of death.

To achieve an accurate estimate of the PMI, the biogenic amines must be separated successfully when integrating HPLC techniques. Successful separation includes baseline separation of each target analyte while maintaining a gaussian peak shape. Many factors can be considered when adjusting a method to achieve separation such as mobile phase composition, mobile phase gradient, and columns. In the case of this research, cadaverine and histamine coeluted resulting in developing a method that not only was useful in identifying the biogenic amines, but

also separating them so that quantification was possible. Various C18 columns including Avantor Prevail Select, Thermo Fisher BDS Hypersil™, Waters Atlantis T3 and Phenomenex Kinetex XB were used to determine the best column to achieve separation between cadaverine and histamine. The Phenomenex Kinetex XB column was proven to have the best separation between cadaverine and histamine standards, while still maintaining sharp gaussian peak shapes. Once separation was achieved for cadaverine and histamine, gradient elution was employed to create a method for the HPLC that achieved separation for each of the biogenic amines used in this research. Additionally, the Phenomenex column allowed the run time to be decreased from 30 minutes total to 18 minutes. Therefore, the newly developed method using the Phenomenex Kinetex column with a gradient elution of 0.1 M Ammoniums Acetate and Acetonitrile as mobile phases was used in determining the concentration of biogenic amines in the vitreous humor.

Biogenic amine research that utilizes porcine vitreous humor establishes biological conditions comparable to the vitreous humor in humans, which in turn can provide insight to how this method can be used in human vitreous humor. These patterns were a result of meticulous HPLC separation and UV/VIS detection method development. Through successful HPLC separation, methylamine, putrescine, cadaverine, and histamine were quantified in porcine vitreous humor. The eyes were decomposed in three different environmental conditions: (a) eyes were decomposed in bags immediately after collection (b) eyes were frozen for a three-day period, then bagged during the decomposition process (c) eyes there were decomposed in a polyurethane box. These four key biogenic amines were analyzed in virtuous humor under these environmental conditions. Each of these conditions allowed the formation of biogenic amines in the vitreous humor. The frozen eyes and the bagged eyes resulted in collection of the vitreous humor over an 8 day period, which extends the analysis period for the potassium concentration in vitreous humor

by three days. The boxed eyes were also able to extend the analysis period of the vitreous humor by one to two days depending on the biogenic amine, when compared to the potassium concentration PMI method. Notably, methylamine, putrescine, and cadaverine exhibit promising patterns in concentration over the 8-day period that can be beneficial to forensic teams in determining the PMI. For each condition the eyes were decomposed in, the trend in concentration of methylamine and putrescine begin to form at a slow rate, then over the course of a day have a significant increase in concentration then plateau. Although both the frozen and bagged eyes have significant methylamine, putrescine and cadaverine concentrations, the bagged eyes have an increased formation threshold than the frozen eye and boxed eyes. However, this was not true for histamine. The frozen eyes have a significant increase in histamine formation than the bagged eyes. This can be attributed to the possible introduction of N-methyltransferase into vitreous humor which could inhibit the production of histamine. Other than methylamine and putrescine, the boxed eyes did not establish a predictable pattern that could be beneficial to estimation of the PMI.

For a more accurate pattern determination, an increase in eye replication will mitigate for any impact outlier data has on overall results. Vitreous humor samples were collected in triplicate meaning that there were three separate eyes used for collection each day; however, increasing the replication per day is warranted to increase precision of the average biogenic amine concentrations, which in turn, will allow for a more accurate idea of the pattern the formation of the biogenic amines follow. Furthermore, experimentation can be conducted on the formation of aldehydes in the vitreous humor. Exploring the relationship between biogenic amine formation and aldehyde formation can give another method to estimate the PMI. With the introduction of water, amine oxidase of the biogenic amines occur and results in the formation of aldehydes. Theoretically, a ratio between the aldehyde formation and the biogenic amines can be used to get

a deeper understanding of chemical processes that occur during decomposition which can be used to further shorten the PMI estimate. Integrating these insights into forensic practices will refine PMI estimation so that forensic teams are able to get a more accurate estimation of the duration a cadaver has been decaying.

REFERENCES

(1)   Franceschetti, L.; Amadasi, A.; Bugelli, V.; Bolsi, G.; Tsokos, M. Estimation of Late Postmortem Interval: Where Do We Stand? A Literature Review. *Biology* **2023**, *12* (6). https://doi.org/10.3390/biology12060783.

(2)   Dix, J.; Graham, M. *Time of Death, Decomposition, and Identification : An Atlas*; Causes of death atlas series.

(3)   Madea, B.; Rödig, A. Time of Death Dependent Criteria in Vitreous Humor—Accuracy of Estimating the Time since Death. *Forensic Sci. Int.* **2006**, *164* (2), 87–92. https://doi.org/10.1016/j.forsciint.2005.12.002.

(4)   Snell, R. S.; Lemp, M. A. *Clinical Anatomy of the Eye*; Wiley, 2013.

(5)   Chronister, C. W.; Walrath, J. C.; Goldberger, B. A. Rapid Detection of Benzoylecgonine in Vitreous Humor by Enzyme Immunoassay*. *J. Anal. Toxicol.* **2001**, *25* (7), 621–624. https://doi.org/10.1093/jat/25.7.621.

(6)   Shukla, S.; Park, H.-K.; Kim, J.-K.; Kim, M. Determination of Biogenic Amines in Korean Traditional Fermented Soybean Paste (Doenjang). *Food Chem. Toxicol.* **2010**, *48* (5), 1191–1195. https://doi.org/10.1016/j.fct.2010.01.034.

(7)   Ladero, V.; Calles-Enriquez, M.; Fernandez, M.; Alvarez, M. A. Toxicological Effects of Dietary Biogenic Amines. *Curr. Nutr. Food Sci.* **2010**, *6* (2), 145–156. https://doi.org/10.2174/157340110791233256.

(8)   Boumba, V. A.; Ziavrou, K. S.; Vougiouklakis, T. Biochemical Pathways Generating Post-Mortem Volatile Compounds Co-Detected during Forensic Ethanol Analyses. *Forensic Sci. Int.* **2008**, *174* (2), 133–151. https://doi.org/10.1016/j.forsciint.2007.03.018.

(9)   Proestos, C. *Biogenic Amines*; IntechOpen, 2019.

(10)   Wishnok, S.; Blusztajn, K. Formation of Methylamines from Ingested Choline and Lecithin. *225*.

(11)   Yang, S.; Mu, L.; Feng, R.; Kong, X. Selection of Internal Standards for Quantitative Matrix-Assisted Laser Desorption/Ionization Mass Spectrometric Analysis Based on Correlation Coefficients. *ACS Omega* **2019**, *4* (5), 8249–8254. https://doi.org/10.1021/acsomega.9b00566.

(12)   Arsenault, J. C.; McDonald, P. D. *Beginners Guide to Liquid Chromatography*; Waters Corporation: Milford, Mass, 2009.

(13) Žuvela, P.; Skoczylas, M.; Jay Liu, J.; Bączek, T.; Kaliszan, R.; Wong, M. W.; Buszewski, B. Column Characterization and Selection Systems in Reversed-Phase High-Performance Liquid Chromatography. *Chem. Rev.* **2019**, *119* (6), 3674–3729. https://doi.org/10.1021/acs.chemrev.8b00246.

(14) Snyder, L. R.; Kirkland, J. J.; Dolan, J. W. *Introduction to Modern Liquid Chromatography*; Wiley, 2011.

(15) Horváth, C. *High-Performance Liquid Chromatography: Advances and Perspectives*; Advances and Perspectives; Elsevier Science, 2013.

(16) Casoni, D.; Sârbu, C. Comprehensive Evaluation of Lipophilicity of Biogenic Amines and Related Compounds Using Different Chemically Bonded Phases and Various Descriptors. *J. Sep. Sci.* **2012**, *35* (8), 915–921. https://doi.org/10.1002/jssc.201101032.

(17) Swartz, M. HPLC DETECTORS: A BRIEF REVIEW. *J. Liq. Chromatogr. Relat. Technol.* **2010**, *33* (9–12), 1130–1150.

(18) David, V.; Moldoveanu, S. C.; Galaon, T. Derivatization Procedures and Their Analytical Performances for HPLC Determination in Bioanalysis. *Biomed. Chromatogr.* **2021**, *35* (1), e5008. https://doi.org/10.1002/bmc.5008.

(19) Jain, A.; Verma, K. K. Strategies in Liquid Chromatographic Methods for the Analysis of Biogenic Amines without and with Derivatization. *TrAC Trends Anal. Chem.* **2018**, *109*, 62–82. https://doi.org/10.1016/j.trac.2018.10.001.

(20) Grace Division. Prevail™ HPLC Columns, 2006. https://www.hplc.eu/Downloads/Alltech_Prevail_HPLC_Columns.pdf (accessed 2024-04-08).

(21) Atlantis T3 Column. https://www.waters.com/nextgen/us/en/shop/columns/186003729-atlantis-t3-column-100a-3--m-46-mm-x-150-mm-1-pk.html (accessed 2024-04-08).

(22) Kinetex XB-C18 Core Shell. https://www.phenomenex.com/Products/Kinetex-hplc-column/kinetex-xb-c18#order.

(23) Thermo Fisher. Hypersil BDS HPLC Columns, 2018. https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FCMD%2FSpecification-Sheets%2Fps-21875-hypersil-bds-hplc-columns-ps21875-en.pdf (accessed 2024-04-08).

(24) Naganuma, F.; Nakamura, T.; Yoshikawa, T.; Iida, T.; Miura, Y.; Kárpáti, A.; Matsuzawa, T.; Yanai, A.; Mogi, A.; Mochizuki, T.; Okamura, N.; Yanai, K. Histamine N-Methyltransferase Regulates Aggression and the Sleep-Wake Cycle. *Sci. Rep.* **2017**, *7* (1), 15899. https://doi.org/10.1038/s41598-017-16019-8.

(25) Buis, R. C.; Rust, L.; Nizio, K. D.; Rai, T.; Stuart, B. H.; Forbes, S. L. Investigating the Sensitivity of Cadaver- Detection Dogs to Decomposition Fluid. *J. Forensic Identif.* **2015**, *65* (6), 985–997.