REVERSED-PHASE LIQUID CHROMATOGRPAHY FOR SEPERATION OF ILLICIT DRUGS UTILIZING WATER-RICH MOBILE PHASES

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Comprehensive Chemistry.

BY

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a widely accepted method in drug and toxicological analyses to detect and quantify drugs and metabolites. However, current RP-HPLC methods use mobile phases that contain significant amounts of organic modifiers such as acetonitrile. Although these organic modifiers are effective, they have been linked to negative environmental effects. The focus of this research is to develop a water-rich mobile phase system composed of >90% water and a smaller amount of a nonpolar organic modifier. Long-chain alcohols such as 1-butanol have proved to be suitable organic modifiers for water-rich RP-HPLC analysis. The use of 1-butanol as an organic modifier is studied in the current research to analyze drugs belonging to amphetamine-related drug classes. A series of differing gradients and elution tests were run to observe which method produced the best separation and resolution among the compounds. The preliminary samples include quinine caffeine, vanillin, and acetylsalicylic acid. An isocratic elution using mobile phases containing 0.1% formic acid and butanol in water was able to separate the preliminary sample. The retention times are 1.775, 4.96, 9.769, and 13.373, respectively; additionally, the resolution values are 1.13, 2.53, 2.72, and 1.86. The illicit substances include amphetamine, methamphetamine, and MDEA. An isocratic elution using mobile phases containing a phosphate buffer, pH-adjusted to 7.0 with a 3.5% butanol in water has produced the efficient peak resolution in an illicit drug sample. The retention times are 7.208, 7.932, and 10.053, respectively. A Waters: Atlantis T3 column helped to achieve the separation but could not fully prevent peak tailing. The peak tailing was caused by silanol effects that occur in the stationary phase, but efforts have been made to correct

those effects. However, with the minimal peak tailing, the peaks still achieved a resolution of 1.52, 1.74, and 4.50 which meets the HPLC drug analysis requirement. Overall, this method was able to utilize a water-rich mobile phase with a small amount of organic modifier to separate the amphetamine-related compounds in a mixed sample.

CHAPTER II - INTRODUCTION

Background

Forensic chemistry is a specialized topic, but it combines principal chemistry theories and ideas to solve criminal problems. As society continues to develop, the need for safe and cost-efficient evidence analysis is needed as the number of drug cases increase[1]; crime labs need a way to analyze that evidence quickly and efficiently. Evidence is analyzed at the local and national level and can contain a myriad of samples; thus, forensic labs have the obligation to evaluate all the evidence received. Chromatographic techniques such liquid chromatography mass spectrometry (LC-/MS) and gas chromatography (GC-MS) are commonly used in forensic drug analysis and toxicological analysis to detect and quantify drugs and metabolites. Liquid chromatographic techniques such as HPLC are gaining popularity among forensic drug and toxicological analysis due to several reasons. They do not operate at high temperatures compared to GCMS and this would facilitate the analysis of thermally unstable drug molecules and metabolites. These methods can easily be combined with tandem mass spectral systems to achieve better limit of detections compared to GCMS systems.

High Performance Liquid Chromatography (HPLC)

In any chromatographic system, two distinct phases are used to achieve a separation of compounds (or solutes) in a mixture. The mobile phase moves, as the name suggests, and carries the solutes with it. The stationary phase, that does not move and is

typically present in a column in an HPLC system, makes interactions with the solutes when the mobile phase carries it through the column. Modern HPLC columns are comprised of tightly packed, micrometer size pellicular or porous silica particles. In general, a liquid chromatography instrument contains seven basic components. The components of a typical HPLC instrument can be seen in Figure 1.[2]

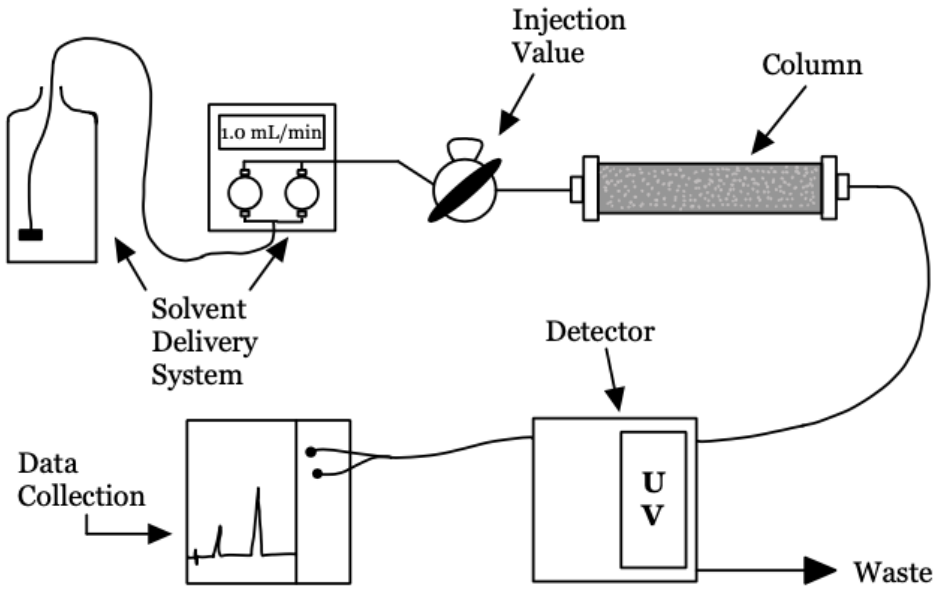

Figure 1: General HPLC diagram

The mobile phase reservoir paired with the solvent delivery system that contains a pump or multiple pumps is used to force the mobile phase through the column. By using one pump and a mixing valve, multiple mobile phase solvents can be used simultaneously. An injection valve is used for sample introduction. A column packed with micrometer size silica particles is used to separate individual compounds in a mixture. These columns can contain different stationary phases including C-8, C-18,

amino, cyano, diol, and phenyl. The choice of the stationary phase depends on the application and the solutes that need to be separated, a stationary phase is represented in Figure 2.

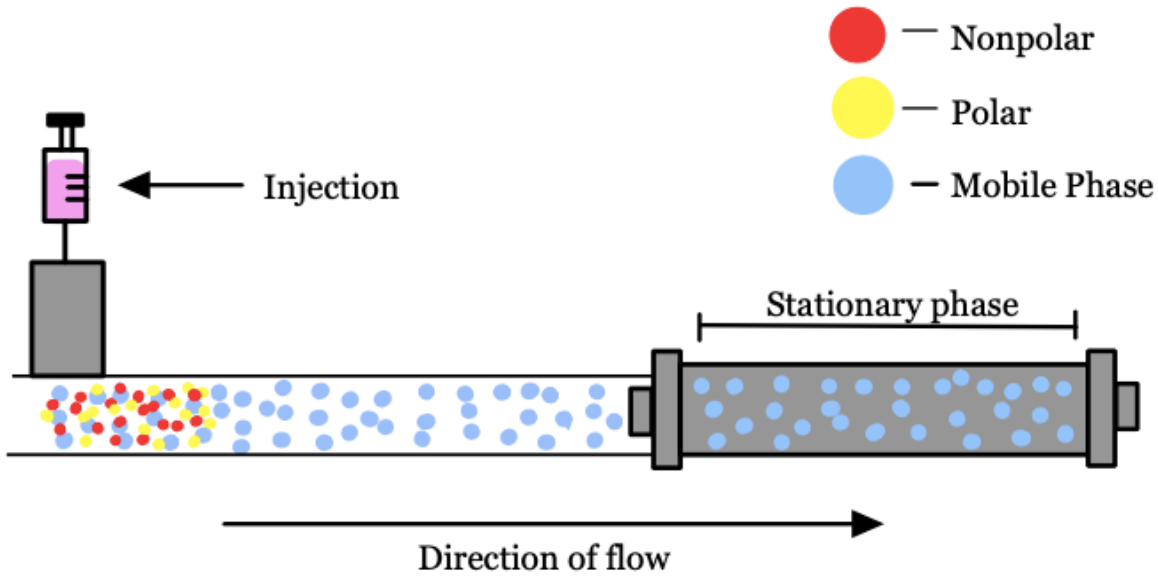

Figure 2: HPLC point of injection and direction of flow through the HPLC system

A detector is used to detect compounds that are eluting from the column. There are several detector types are available and they include refractive index, diode array, fluorescence, mass spectrometry. The most common detectors are diode array detectors, and they operate based on the absorbance of light by molecules in UV/Vis region of the electromagnetic spectrum. There are several types of liquid chromatographic methods. Following is a brief introduction to some of the liquid chromatographic methods.

Bonded-Phase Chromatography

Bonded-phase chromatography, more commonly known as normal-phase and reversed-phase chromatography, utilizes a high-pressure system that allows for the stationary phase to be bonded to a solid surface. [3] The stationary phase is comprised of silica particles that interact with the analyte, and the interactions produce separations in the chromatogram. Normal-phase chromatography uses a polar stationary phase and has a nonpolar mobile phase to incite separations; contrastingly, reversed-phase chromatography uses a nonpolar column with a polar mobile phase (Figure 3 & 4). Therefore, the way analytes navigate the columns is based on the polarity, which can be influenced by the mobile phase, pH, and solubility; thus, the mobile phases can be optimized for specific experiments. [4]

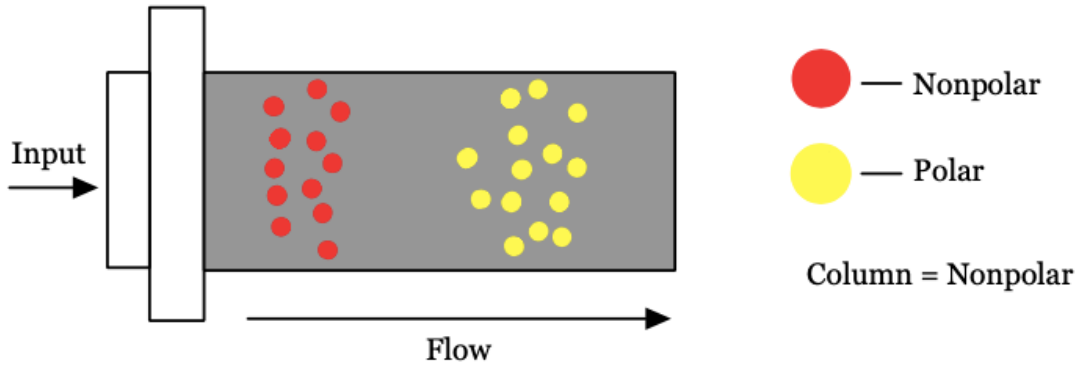

Figure 3: Model of analyte interaction with a nonpolar column.

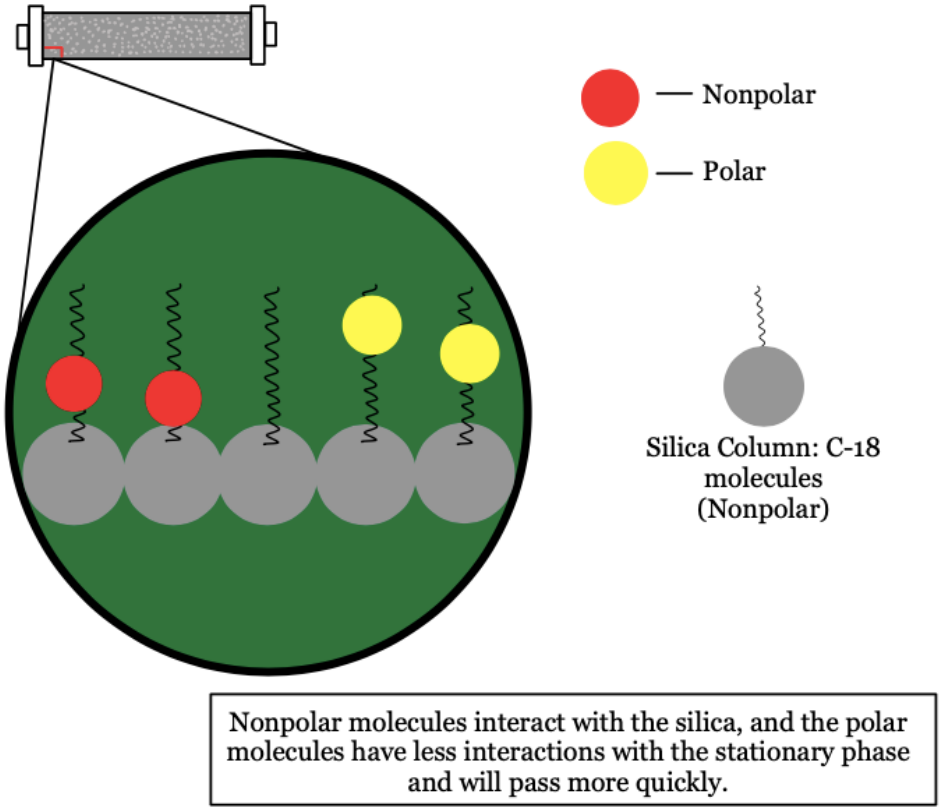

Figure 4: Molecular schematic of analyte interaction.

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is a technique that utilizes charged ions to induce separation in mixtures. Charged ions in the solute compete with the ion in the mobile phase for ionic (retention) sites on the stationary phase. Therefore, the ionic form of the sample is vital to the analysis, so the mobile phase pH may have to be adjusted to accommodate for the form. The ion exchange contains anionic and cationic exchanges; thus, anion exchange column produces cation results and vice versa. Much research has

been conducted with this instrument, and the ease of use for chemicals as well as protein and enzyme applicability have led to it becoming a very versatile LC technique [3,5].

Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) is a method of separation based on when the analyte interacts with a hydrophilic stationary phase in the column. This technique is useful for separation due to the nature of many analytes having the tendency to be hydrophilic or polar. Although, this type of chromatography utilizes a mobile phase that has a high organic content, and commonly uses silica materials that ideal for those conditions. Essentially, this type of chromatography is a normal-phase type of separation, but it uses reversed-phase eluents; methods using this procedure can analyze compounds in complex systems which can aid in many chemical and biochemical systems. [6]

Water-rich Mobile Phases

In a typical HPLC analysis, a mobile phase consists of an organic modifier such as methanol or acetonitrile at a higher concentration. This amount can be varied and can be up to 20% of a more polar solvent as the modifier. In recent studies, Gamagedara et al. and Lavine et al. has shown the use of water-rich mobile phases that contain only a very small amount of long chain alcohols as the organic modifier. [7,9] In one study creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid were able to separate analytes using only 0.1% butanol in water. [8] In another study, imidacloprid and its degradation products were successfully separated using 0.4% of pentanol as the organic modifier.[10] Additionally, Lavine and coworkers were able to separate vanillin and related compounds

using 3.75% 1-butanol in water at pH of 4.07. [9] These studies show that the selectivity of the method is improved by using pentanol or butanol as the organic modifier.

Research Problem

Characteristically, in forensic crime labs, mobile phases used in HPLC for drug analysis utilize a considerable amount of acetonitrile or methanol, that is used as an organic modifier to induce separation.[13] There have been many studies to prove that those methods that use acetonitrile are effective, but over the last five years, the amount of acetonitrile has grown scarce and become expensive, and its volatility has been linked to negative environmental factors.[14] Since the waste generated by these analytical procedures contains a large amount of organic solvents, more rigorous waste disposal methods are necessary. Additionally, both solvents are flammable and have adverse health effects to humans. Thus, development of a more environmentally friendly and safer HPLC methods to be used in forensic laboratories are in need.

Drug Selection

The United States Drug Enforcement Administration (DEA) created a schedule system to identify and sort a myriad of drugs, substances, and chemicals. This system helps to classify the drug's medical ability and the drug's dependency potential. According to the DEA, amphetamine-related drugs fall under schedule II drugs. [(7)] These drugs typically have a high potential for abuse and can lead to physical dependance. Amphetamines are a class of drug known as stimulants which can be prescribed for ADHD and other medical conditions. [11] Therefore, the drug samples were chosen for

analysis based off their molecular structure (Figure 5). They all include the amphetamine backbone which helps to classify them but are not identical; the differing functional groups will allow for the elution to be different for each compound, so ideally separations should be plausible.

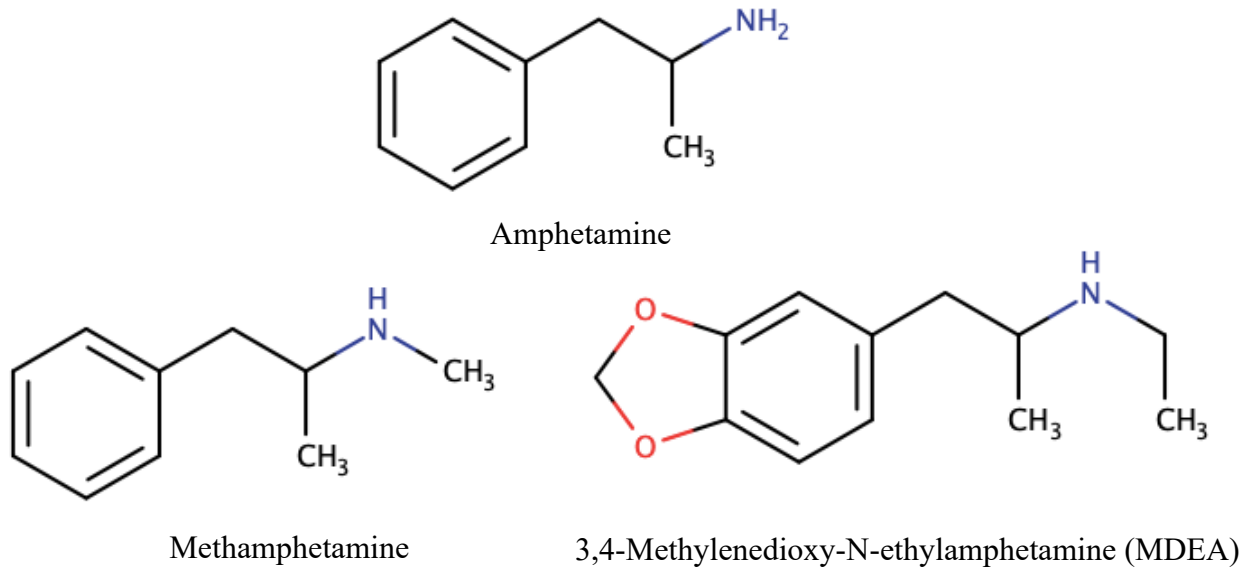

Figure 5: Molecular structures for amphetamine, methamphetamine, and MDEA.

Motivation

RP-HPLC has been a widely accepted method in drug analysis, from crime labs to pharmaceutical research.[12] This instrument separates components of a given sample through hydrophobic interactions. Separation can be influenced by several factors including column type, mobile phase composition, and temperature. Frequently, the RP-HPLC uses a C-18, C-8, C-4, or C-30 column to encourage interactions. The columns can differ depending on what specific interaction is being observed, which can contain polar modified phases or can be phenyl based. Although, depending on the type of sample, the

mobile phases, detector, and the gradient will be modified to get the proper separation. The mobile phase percentages can be altered to allow the eluent to flow at the same rate (isocratic) or change rates over time (gradient) allowing for more or less separation to occur.

The goal of this research is to develop a water-rich mobile phase system that uses more than 90% water and a comparatively smaller amount of a more nonpolar organic modifier such as butanol for drug analysis. 1-butanol was used as the organic modifier because of its potential to have proton donor and proton acceptor interactions, which can be attributed to separation of different compounds in the column. However, polar compounds such as methamphetamine and other amphetamine related compounds come with its own challenges. These polar compounds are insoluble in water, and these solubility issues can negatively affect the interaction with the silica column. This effect on the separation can be attributed to silanol effects. The silanol effects occur in ionic compounds, especially in basic samples like amphetamine and methamphetamine, which can cause increased retention, peak tailing, and irreproducibility. [14] Generally, these effects occur based on an ion exchange, but can be remedied through sample specific column selection and pH adjustments. Therefore, this method will manipulate the mobile phases to introduce 1-butanol as an organic modifier as well as adapt the RP-HPLC conditions to observe if successful separation among an illicit drug sample is possible.

CHAPTER THREE - MATERIALS & INSTRUMENTATION

Preliminary chemicals: quinine, caffeine vanillin, and acetylsalicylic acid

Vanillin was obtained from the Aldrich Chemical company, acetylsalicylic acid was obtained from Acros Organics, and caffeine and quinine sulfate were obtained from Fisher Scientific Company.

Illicit drug samples: methamphetamine, amphetamine, and 3,4-Methylenedioxy-N-ethylamphetamine (MDEA):

All the controlled substances all purchased from Cerilliant; These samples are used for analytical standards for drug analysis with a concentration of 1mg/mL.

Storage Conditions:

All samples were stored in a -20 ^{o}C freezer until needed for testing.

Mobile phases:

The mobile phases were comprised of HPLC grade formic acid, 1-butanol, dibasic sodium phosphate and ultra-pure water. The formic acid, 1-butanol, and dibasic sodium phosphate were obtained from Fisher Chemical and the ultra-pure water was obtained from Millipore Simplicity equipment.

Instrumentation:

The samples were analyzed using an Agilent 1220 LC. The injection volume was 5µL, the flow rate was a constant 1.0 mL/min, the wavelength was set at 220 nm, the run was 25 minutes per sample, and all methods were conducted using an isocratic elution.

CHAPTER FOUR: PROOF OF CONCEPT

Experimental

The first RP-HPLC method was developed by focusing on the chemical makeup of the mobile phases, specifically how 1-butanol could be related to previous research utilizing acetonitrile. To do this, the polarity index (PI) was calculated for acetonitrile, and then compared to the polarity index of 1-butanol. [11] Polarity index is defined by Snyder as the ability of a solvent to interact with various test solutes and the polarity index of a mixture of solvents can be calculated by using Equation 1. [24]

$$P^1 = \emptyset_a P_a + \emptyset_b P_b \qquad (1)$$

Where P^1 is the polarity index of the mixture of solvents A and B that have polarity indices of $P_a$and P_b respectively. $\emptyset_a$ and $\emptyset_b$ are volume fractions of solvents A and B in the solvent mixture. To determine the amount of butanol needed to have a mobile phase that has similar polarity index to a commonly used mobile phase system that uses acetonitrile, a calculation was made using equation 1. Most of the methods that were reported for drug analysis used a varying amount of acetonitrile in mobile phases and here, the concentration of acetonitrile was set to 20% as starting point.

Acetonitrile Polarity Index Calculation*:

$$ACN\ PI = (0.8 * 9.0) + (6.2 * 0.2)$$

$$PI = 8.44$$

Acetonitrile/1-butanol Comparison*:

$$8.44 = 3.9x + ((1 - x) * 9)$$

$$x = 0.1098 \sim 10\%$$

*Polarity indices - ACN: 6.2, 1-butanol: 3., Water: 9.0, Formic acid is negligible. [24]

The solubility of butanol is around 9% and the mobile was initially made at 7% butanol in water but was later changed to 3.5% to prevent any immiscibility of the solvents during the analysis. [25]

Test Compounds

Caffeine, vanillin, acetylsalicylic acid, and quinine sulfate were collected for preliminary testing with the adjusted mobile phases. They were selected based on the molecular composition and behavior in relation to the controlled substances picked for testing (Figure 6). Additionally, the pKa values are as follows: caffeine = 14.0 , quinine = 4.1 & 8.5, vanillin = 7.43, acetylsalicylic acid = 2.97. [(28-31)]

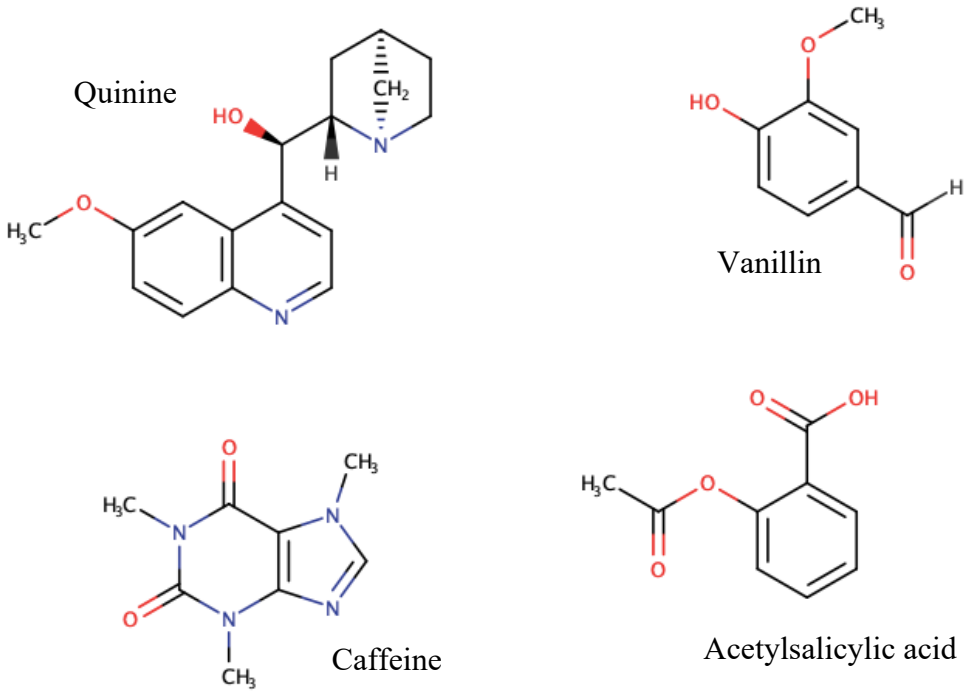

Figure 6: Molecular structures of quinine, vanillin, caffeine, and acetylsalicylic acid.

The combination of the over-the-counter medications and common chemicals allowed for a wide range of data to be collected. Each sample was made into a 0.01 M solution in HPLC grade methanol and analyzed individually with the Alpha mobile phase conditions (Table1).

Table 1: Proof of Concept RP-HPLC Mobile Phase Conditions

Name of Conditions:	**Mobile Phase A**	**Mobile Phase B**	**Bottle Percentages**	**Wavelength (nm)**
Alpha	0.1% Formic acid in water	0.1% Formic acid with 3.5% butanol in water	50:50	220
Beta			30:70	220
Gamma			20:80	220
Delta			0:100	220
Epsilon			0:100	235
Zeta	0.05% Formic acid with 3.5% butanol in water	N/A	100%	220

Preliminary Method Development

These chromatograms show the results based on the conditions in Table1. Each sample was analyzed individually before a mixed sample was created. The mixed sample was comprised of quinine, caffeine, vanillin, and acetylsalicylic acid. These compounds have a wide range of functional groups, and the differing molecular structures which would allow to predict the retention behavior of wide range of compounds. Acidic pH was selected by adding formic acid to the mobile phase as in previous research on water-rich mobile phases showed successful elution of various compounds in acidic conditions. [7-10] The elution order of the compounds was determined by injecting individual compounds using Alpha conditions. The combined sample of those three compounds were mixed and injected to obtain the results. In the next steps, caffeine was introduced as another compound to add more diversity to the test mixture. This revised mixed sample continued to be analyzed using the Beta through Zeta conditions.

Results & Discussion

All the preliminary samples were analyzed individually using the Alpha conditions; caffeine was not included with the initial steps shown in Figure 7. Although the successful separation of all compounds was observed, the vanillin and acetylsalicylic acid had broad peaks. This can be attributed to the longer elution times due to the less nonpolar nature of the mobile phase. In subsequent analysis, butanol concentration was increased to reduce the elution time.

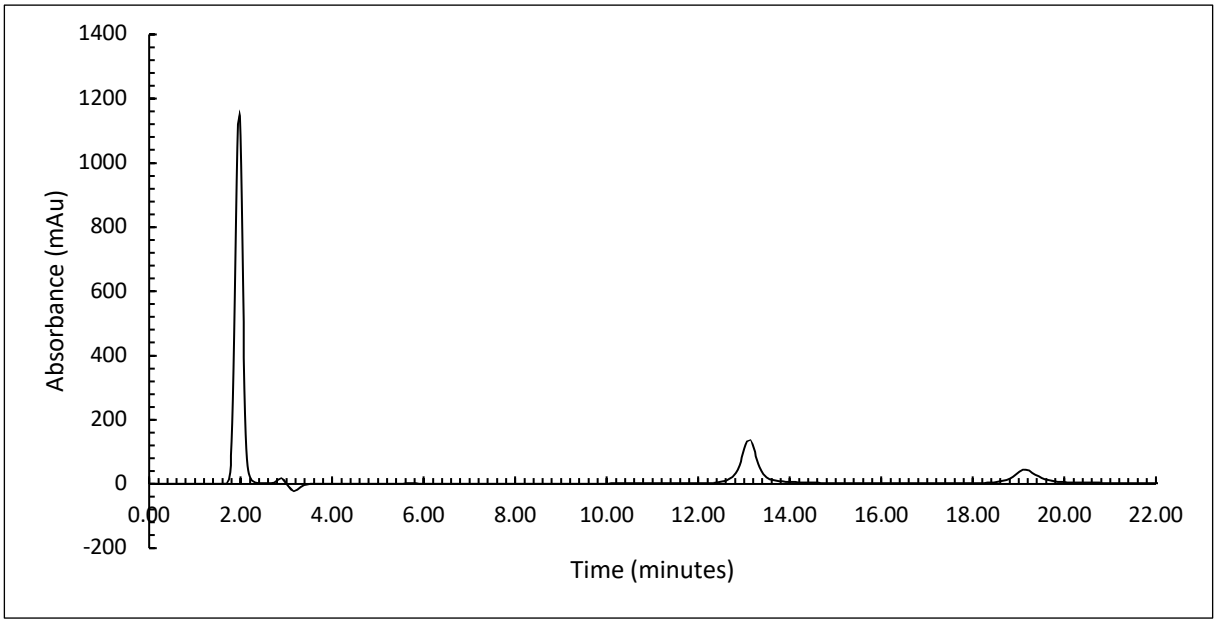

Figure 7: Alpha conditions. This mixed sample included quinine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 2.123, 14.964, and 21.959 minutes, respectively. This sample was analyzed with the Alltech: Prevail column.

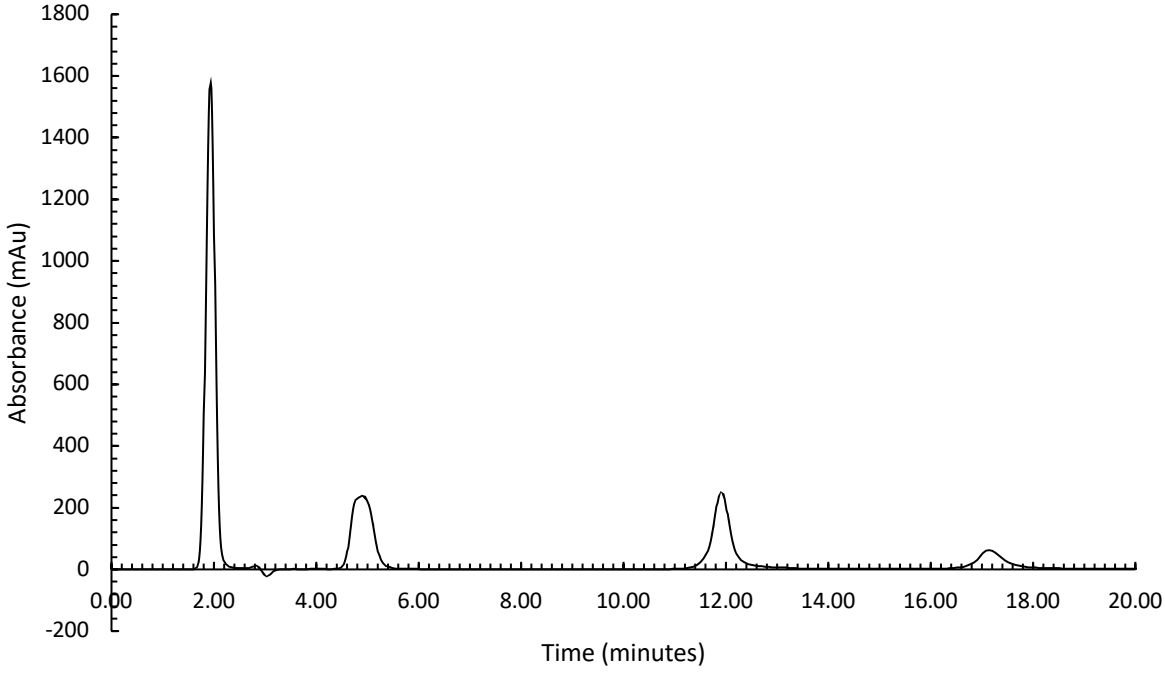

Figure 8: Beta conditions. This mixed sample included quinine, caffeine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 1.894, 4.507, 11.350, and 16.132 minutes, respectively. This sample was analyzed with the Alltech: Prevail column.

The goal of the following analysis was to achieve a shorter elution time of all compounds in the text mixture while maintaining the resolution at an optimum level. Although the resolution can be calculated, here the focus was to achieve baseline separation of the compounds analyzed. It is known that the optimum resolution would include a resolution of 1.5 or greater would be acceptable as a successful separation. [17] Incrementing butanol concentration helped reduce the retention time as seen in Figure 8. The Beta conditions were conducted using the 30:70 isocratic elution, but two things were of concern in this chromatogram (Figure 8). The caffeine peak was broad which could be adapted to have a more gaussian shape. Peak broadening can be caused by longitudinal diffusion across the column. This phenomenon occurs when the band of the compound begins to separate in the column, where the concentration is the greatest in the middle, so the band hits the detector at dispersed times; thus, causing peak broadness outside of the usual random path of diffusion. [27] This was taken into consideration for the Gamma conditions; these conditions included a 20:80 isocratic elution which was successful in fixing the broadness of the caffeine peak as well as maintaining the shape and separation for the other compounds. The Delta conditions produced similar resolutions and separation, but there was a slight difference in the retention times. The Delta retention times show that the elution happened at a quicker rate than the those of the Gamma conditions.

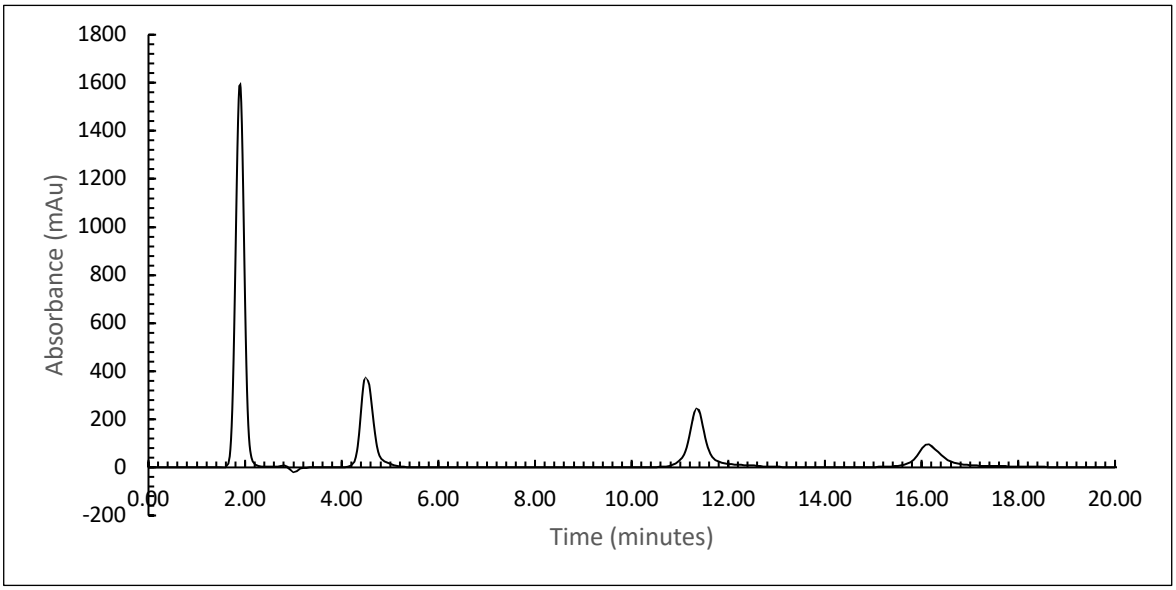

Figure 9: Gamma conditions. This mixed sample included quinine, caffeine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 1.934, 4.907, 11.914, and 17.143 minutes, respectively. The sample was analyzed with the Alltech: Prevail column.

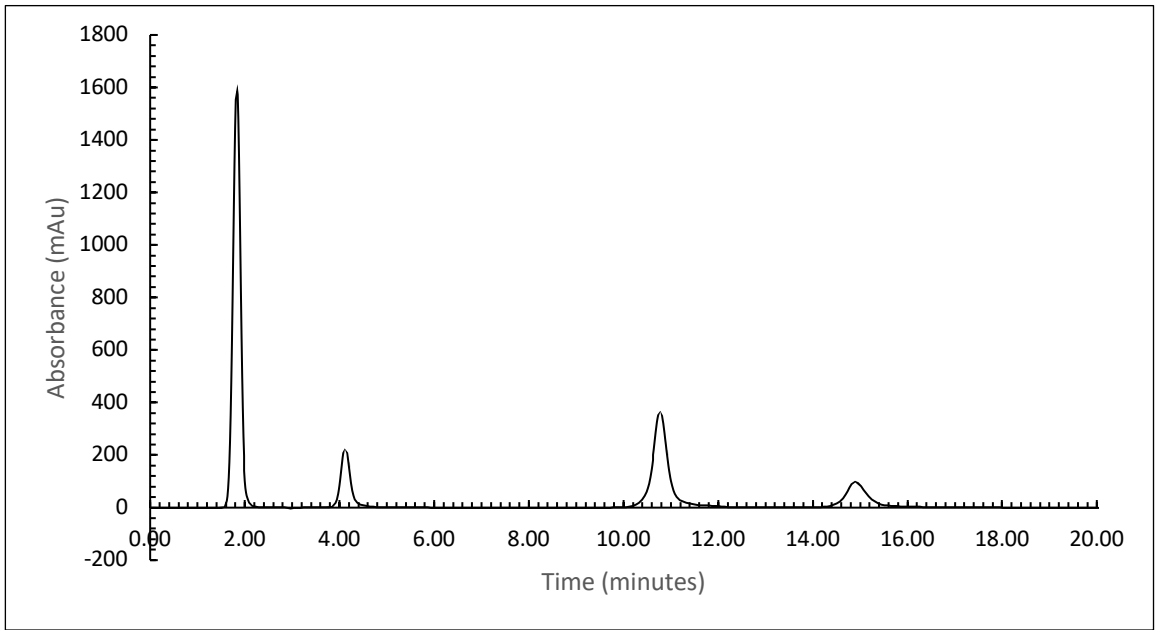

Figure 10: Delta conditions. This mixed sample included quinine, caffeine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 1.823, 4.124, 10.773, and 14.892 minutes, respectively. This sample was analyzed with the Alltech: Prevail column.

This result prompted for the mobile phase conditions to be altered once more by having mobile phase B at 100%. At this condition, butanol was present at 3.5%. Those results produced similar results to the Gamma conditions, the peak shape and retentions were almost identical, so no change occurred (Figure 10). Additionally, there was one more set of conditions that were of interest, and that was manipulating the wavelength of the detector. The detector used has the ability to use only a single wavelength at a time and the detector wavelength was changed from 220 nm to 235 nm.[13] Additionally, the UV absorption at this wavelength tend to be compounds that contain aromatic rings; those rings can have an absorption maximum between 215 and 240 nm, optimizing the wavelength can have an effect on the sensitivity which would increase the ability to detect compounds at small concentrations.[26]

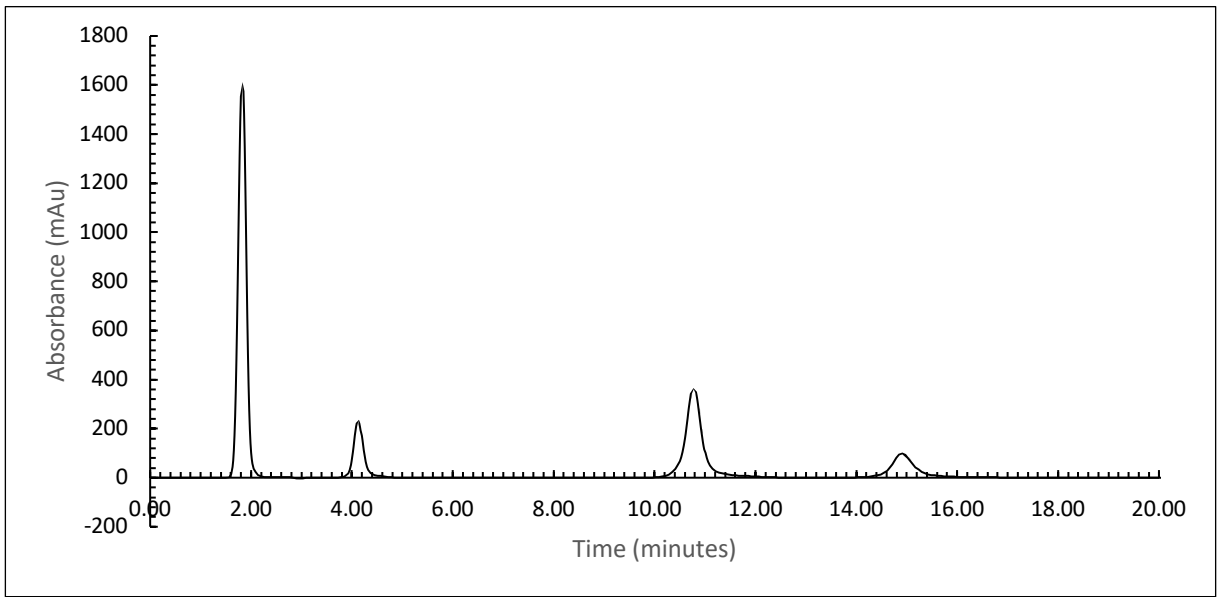

Figure 11: Epsilon conditions. This mixed sample included quinine, caffeine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 1.826, 4.124, 10.776, and 14.90 minutes, respectively. This sample was analyzed with the Alltech: Prevail column.

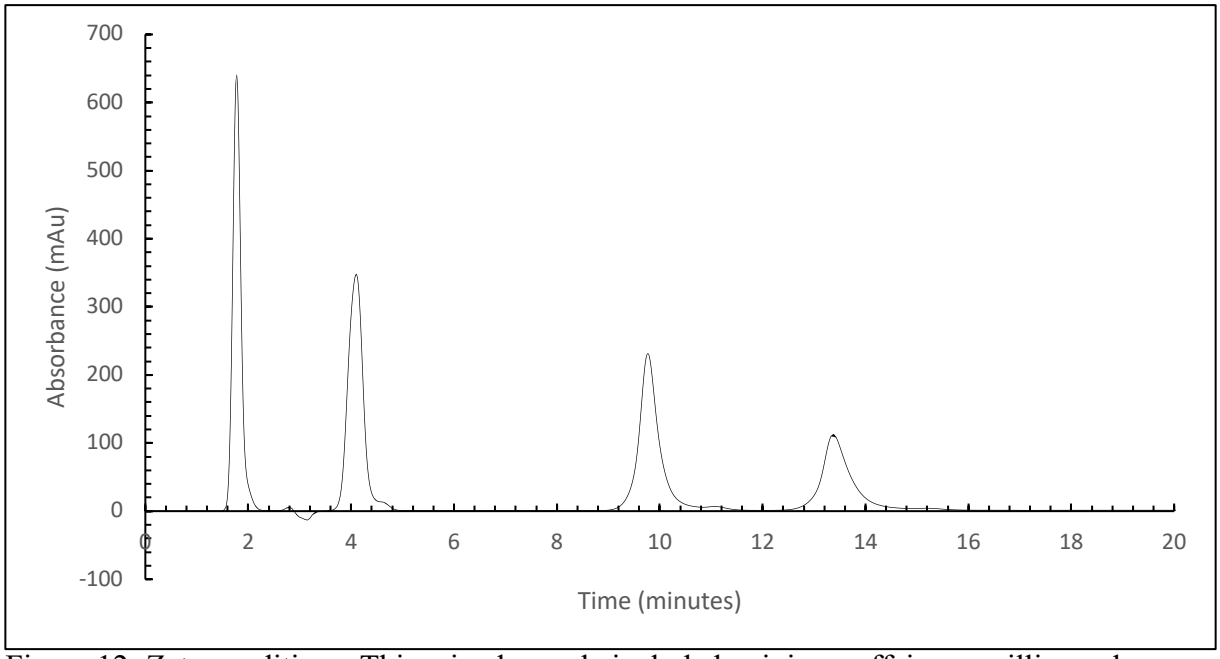

Figure 12: Zeta conditions. This mixed sample included quinine, caffeine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 1.775, 4.096, 9.769, and 13.373 minutes, respectively. This sample was analyzed with the Alltech: Prevail column.

These conditions were imperative for this research as this was intended to amend the elution issue. It was observed that all the compounds had baseline separation. However, the quinine peak was eluting before the solvent peak which can be seen in all of the chromatograms, when the solvent peal is supposed to represent deadtime in the HPLC system. In an effort to fix this issue, the amount of formic acid was decreased by half to 0.05%, but even with the alterations to the method the early elution of quinine is observed. This can be explained using the acid-base equilibria of quinine at this mobile phase conditions. The pH of the mobile phase was at 3.1, due to the presence of formic acid, and this can be attributed to yield more ionized form of quinine compared to un-ionized form due to its basic properties (see Figure 6) This ionized form will interact more with the mobile phase compared to stationary phase. This is predicated to occur from the ionized form of the nitrogen and hydroxyl groups which cause those groups to

elute faster. The next chapter is focused on changing the pH of the mobile phase with amphetamine related compounds and the test compounds used in this chapter was analyzed with all mobile phase conditions that are listed in Table 2. Additionally, these compounds are analyzed with two other HPLC columns (see Table 2.) The results of these injections are not shown here. After optimizing the pH and the column conditions, the elution issue of quinine was fixed, and the resulting chromatograph is shown in Figure 13. Here the peak around 1.8 minutes was found to be for the elution of the solvent.

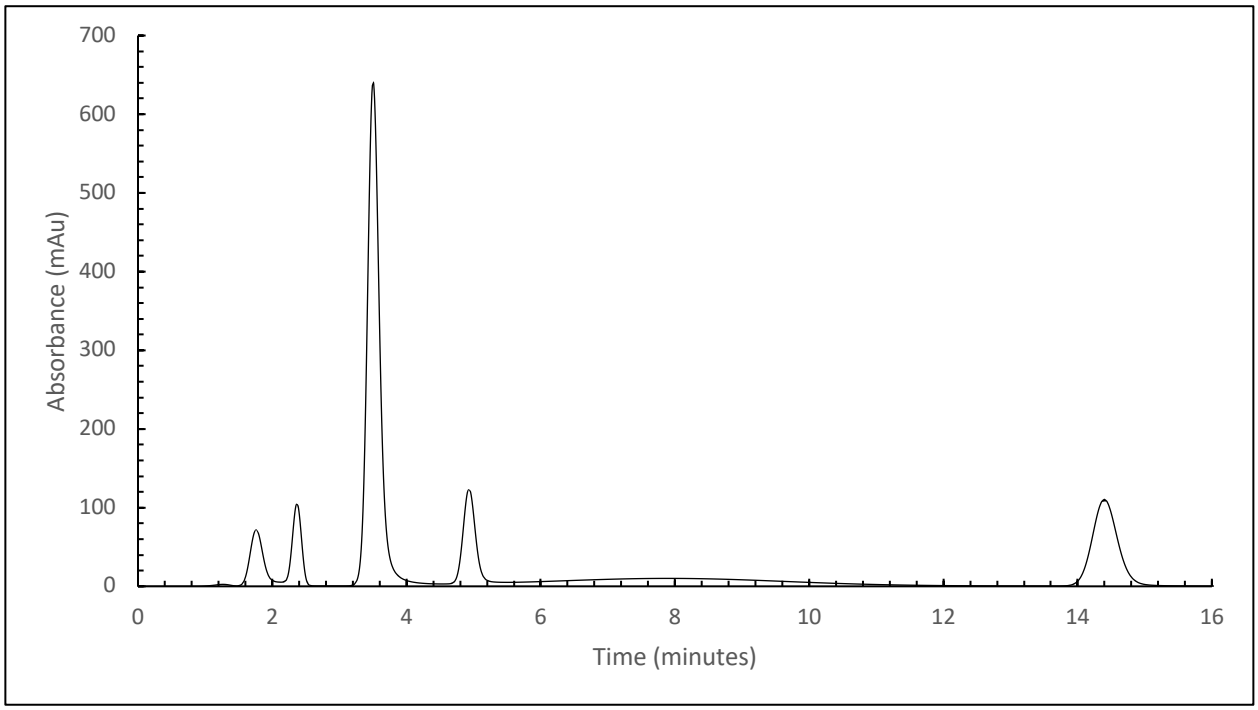

Figure 13: This mixed sample included caffeine, quinine, vanillin, and acetylsalicylic acid, and the retention times of the compounds are 2.368, 3.584, 4.932, and 14.398 minutes, respectively; additionally, the resolution values are 1.13, 2.53, 2.72, and 1.86 The sample was analyzed with the Waters: Atlantis T3 column.

CHAPTER FIVE: ILLICIT SUBSTANCE ANALYSIS

Experimental

This chapter focuses on the analysis of amphetamine related compounds using water-rich mobile phases. Methamphetamine, amphetamine, and MDEA were used due to two reasons: (a) they all share similar structures and using them as the test mixture, a deeper understanding about the retention mechanism in water-rich environment can be studied. (b) These compounds are a part of routine analysis that are conducted in crime labs. The structures of the compounds used can be found in Figure 5. All three compounds have similar structures and basic properties due to the amine groups present. Here the focus was to optimize the pH and the mobile phase composition to achieve the optimum separation of compounds. The pH can be further investigated through the Henderson-Hasselbalch equation to observe the percentages between the ionized and unionized forms of the compounds.

Table 2: RP-HPLC Mobile Phase Conditions

	Mobile Phase A	**Mobile Phase B**	**Percentages (If applicable)**
A	Phosphate buffer with 3.5% butanol at pH of 6.5	N/A	N/A
B	Phosphate buffer with 3.5% butanol at pH of 7.0	N/A	N/A
C	Phosphate buffer at pH of 7.0	Phosphate buffer with 3.5% butanol at pH of 7.0	50:50
D			60:40
E			70:30
F			80:20

Throughout the process, three columns were analyzed to record distinctive column interactions. The column specifications can be seen in Table 3, with differences including column dimension and particle size.

Table 3: Column Specifications

Name	Bonded phase	Column Dimension	Particle size	Pore Size
Alltech: Prevail	C18	4.6 x 250 mm	5 µm	100 Å
Thermoscientific: BDS Hypersil		4.6 x 100 mm	5 µm	130 Å
Waters: Atlantis T3		4.6 x 150 mm	3 µm	100 Å

Illicit Substance Method Development

The test sample was comprised of amphetamine, methamphetamine, and MDEA, and eluted in that order. These compounds have a similar molecular structure with only a few differing functional groups, so these series of tests attempted to separate the compounds considering their similar structure and basic nature. The pKa values are as follows: amphetamine = 9.9, methamphetamine = 9.87, and MDEA = 10.22. [32-34]

The previous research had proven that separation between the compounds in the initial sample was possible, but included an elution issue that was not ideal for the proof of these conditions. Therefore, additional alterations needed to be made to mend this problem. That process started with two components: the addition of a buffer solution and a range of pH tests. This suggested that the buffer could be added and be able to resist changes in pH if any unpredicted reactions were to occur. [15] The alkali buffer contains a weak base and a salt, and this allows for Le Chatelier's principle to be fulfilled; if the

ions in the solution were to shift towards being more acidic or more basic, the buffer should be able to resist that change and balance out at equilibrium. [23] The buffer created was a 50 mM solution that consisted of dibasic sodium phosphate (Na_2HPO_4) and ultrapure water mixture. As seen in Table 2, the buffer was added to the existing butanol percentage, and conditions A and B had a range of differing pHs. The pH can have a significant impact on retention and peak shape. In this research, the organic-aqueous mobile phases depending highly on the solubility of the analyte, and the excessive percentage of water could have a significant influence over retention and pH. The goal of adjusting the pH, in addition to the buffer, was to allow the sample to elute with a delayed retention time to avoid eluting before the solvent peak as seen in the preliminary data. As seen in Table 3, there were two different pH experiments, and those included 6.5 and 7.0 which were the upper level of the column threshold; the pH was adjusted with hydrochloric acid as needed to lower the pH. All the columns used during this research had a pH range of 2-8 and using a pH outside this range could cause long-term harm to the column.[23]

Results & Discussion

Conditions A and B and were analyzed using the Alltech: Prevail column; Figures 14 & 15 show the results of this analysis.

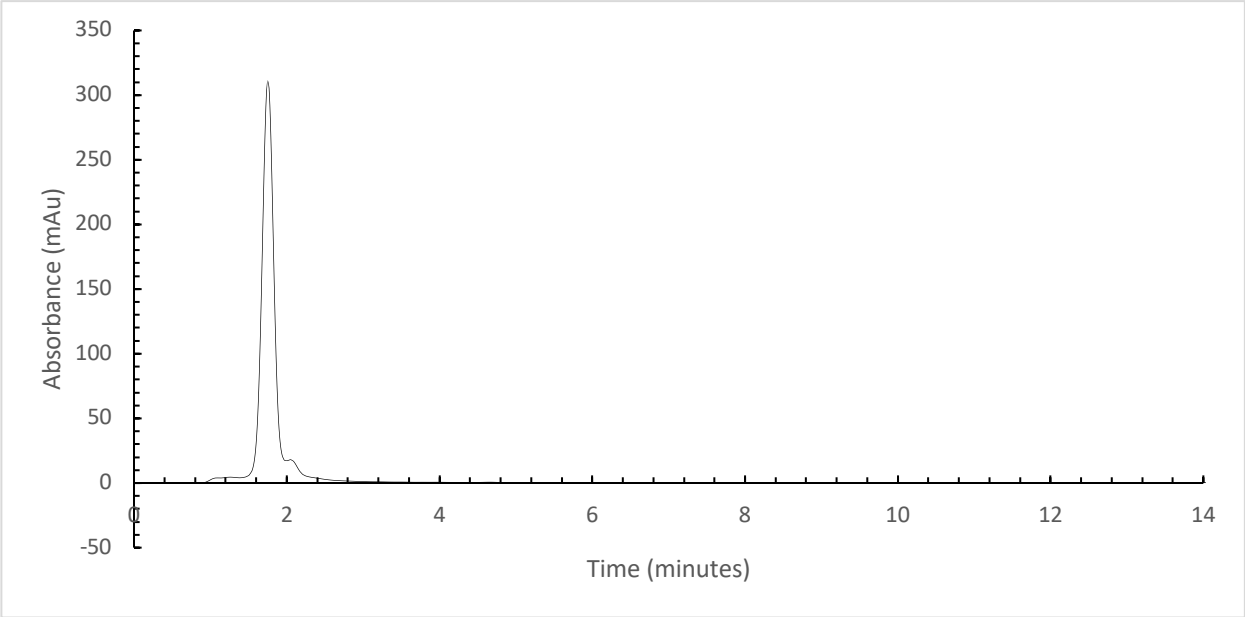

Figure 14: Condition A. This mixed sample included amphetamine, methamphetamine, and MDEA, and all the compounds eluted at the same time. The retention time of the peak is 1.753. This sample was analyzed with the Alltech: Prevail column.

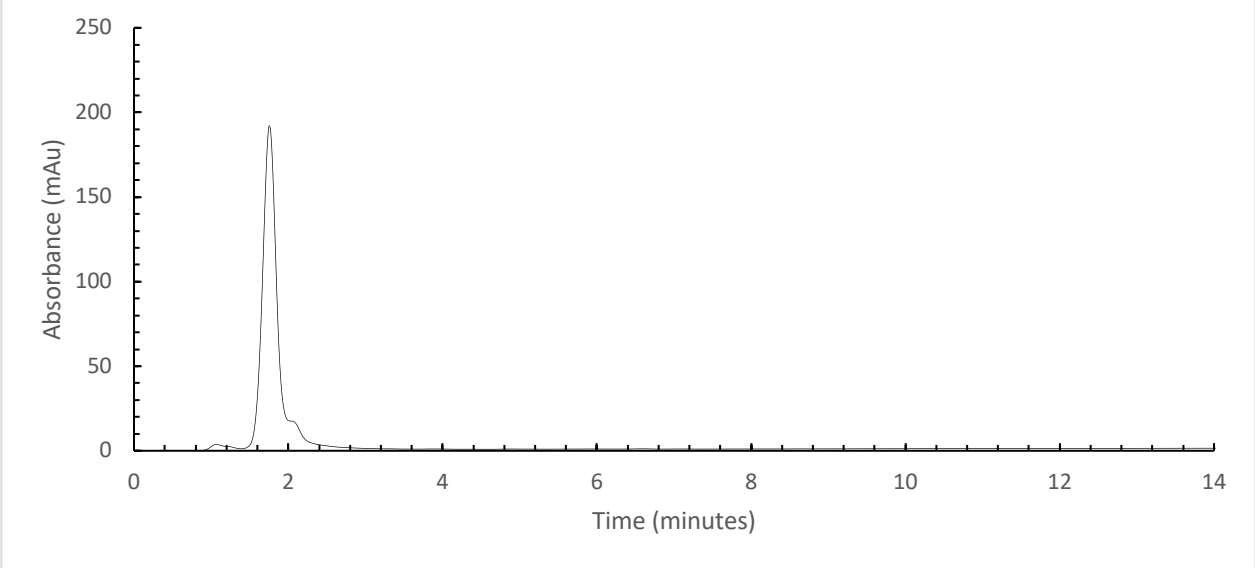

Figure 15: Condition B. This mixed drug sample included amphetamine, methamphetamine, and MDEA, and all the compounds eluted at the same time. The retention time of the peak is 1.756. This sample was analyzed with the Alltech: Prevail column.

Both chromatograms show complete overlap therefore, no differentiation between the samples can be made. At this point, there was no success with the mobile phases, so the next step was to change columns and observe the results. The Alltech: Prevail was

switched with the Thermoscientific: BDS Hypersil. There were two main differences between this column that can be seen in Table3, the length of the column and the pore size; the shorter length reduces the time of the analysis but could cause low resolution, and the larger pore size could be beneficial for larger molecules. With that, individual samples of the illicit samples were analyzed with the 6.5 and 7.0 mobile phases which can be seen in Figures 16 & 17.

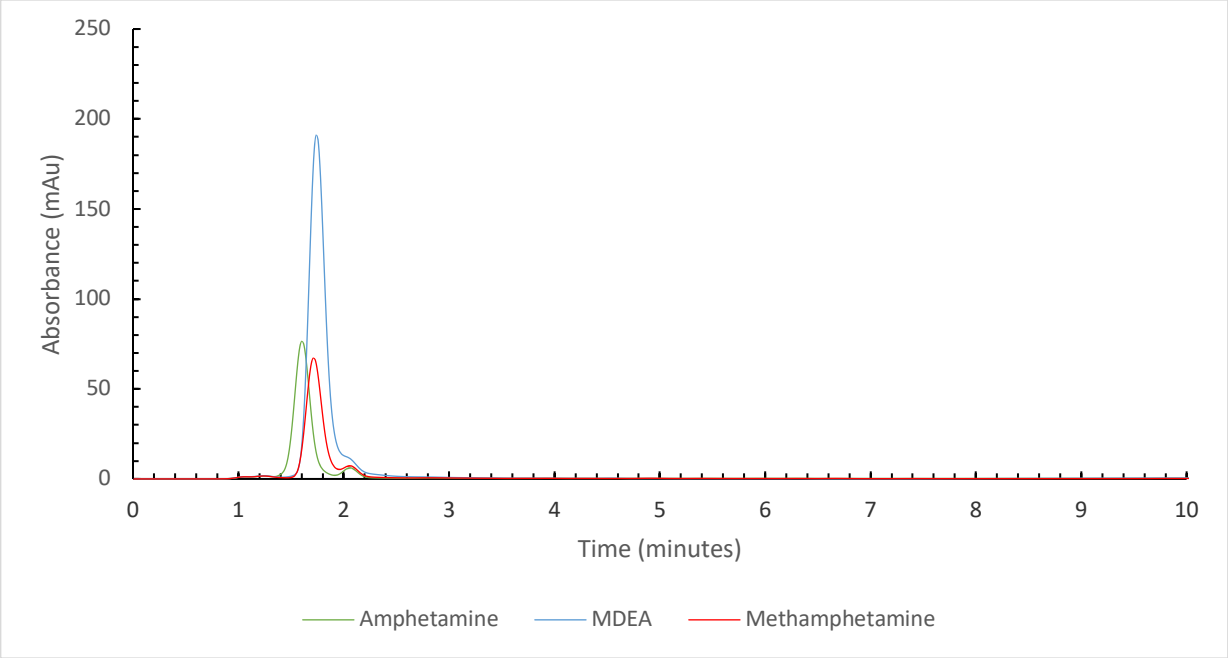

Figure 16: Condition A. This mixed drug sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 1.605, 1.716, and 1.743 minutes, respectively. These samples were analyzed with the Thermoscientific: BDS Hypersil column.

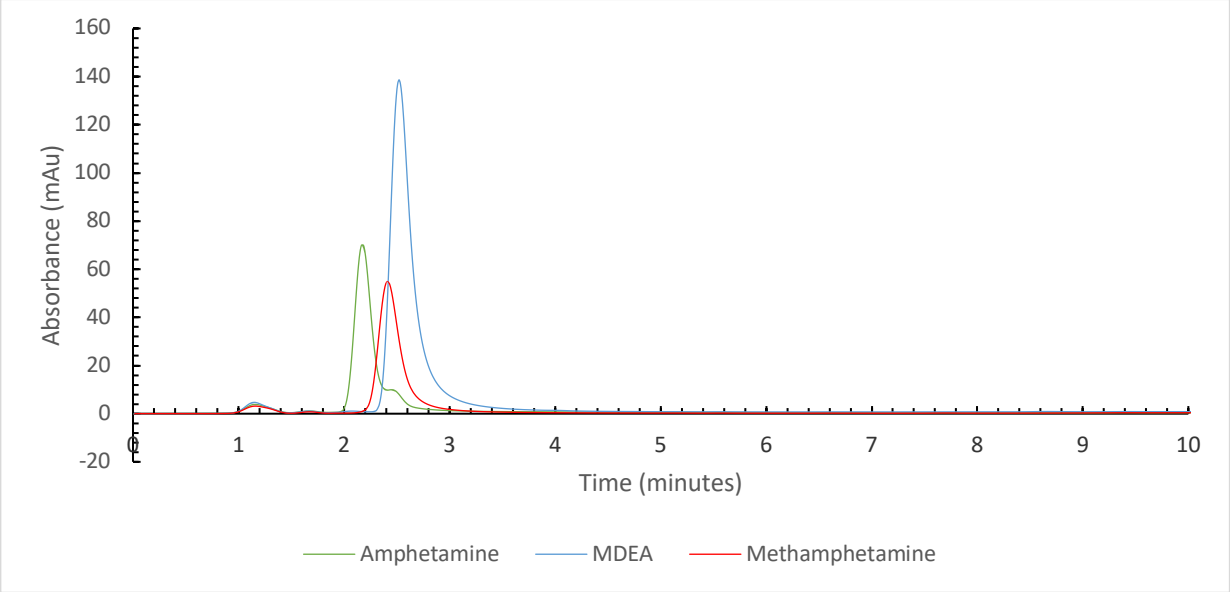

Figure 17: Condition B. This mixed drug sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 2.175, 2.415, and 2.522 minutes, respectively. These samples were analyzed with the Thermoscientific: BDS Hypersil column.

Some separation between compounds was observed at pH 7.0 indicating the acid base equilibrium is shifting towards having more unionized form of the drug molecules. With these results, it is hypothesized that the organic content in the mobile phase is causing the compounds to elute faster. To increase the interaction between the drug molecules and the column, the butanol content was reduced in subsequent analysis (see Table 2).

Based off these results, the peak shape seemed to improve as well as it began to increase separation between the compounds, but looking at the retention times, the 7.0-adjusted mobile phase was able to separate the compounds; this process of analyzing the pH gave enough data to confidently use the 7.0 mobile phase for the remainder of the research. The mobile phases would be run isocratically with a range of percentages using the Thermoscientific: BDS Hypersil column. Specifically, 50:50, 60:40, 70:30 and 80:20, and the data can be seen in Figures 18-21.

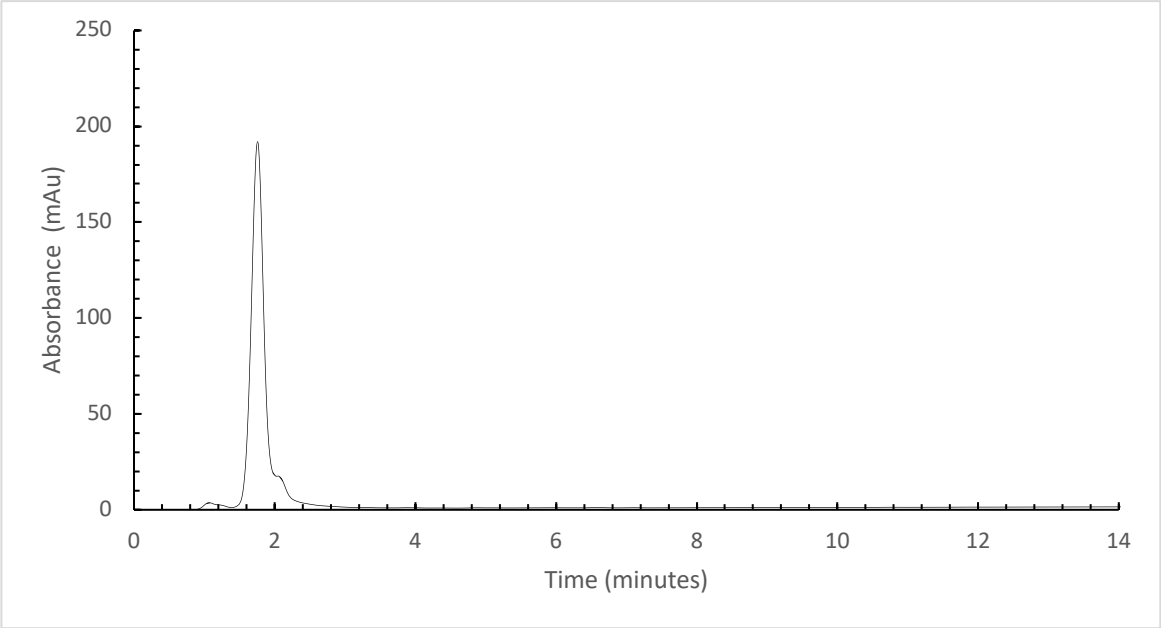

Figure 18: Condition C. This mixed drug sample included amphetamine, methamphetamine, and MDEA. The retention time of the peak is 1.765. These samples were analyzed with the Thermoscientific: BDS Hypersil column.

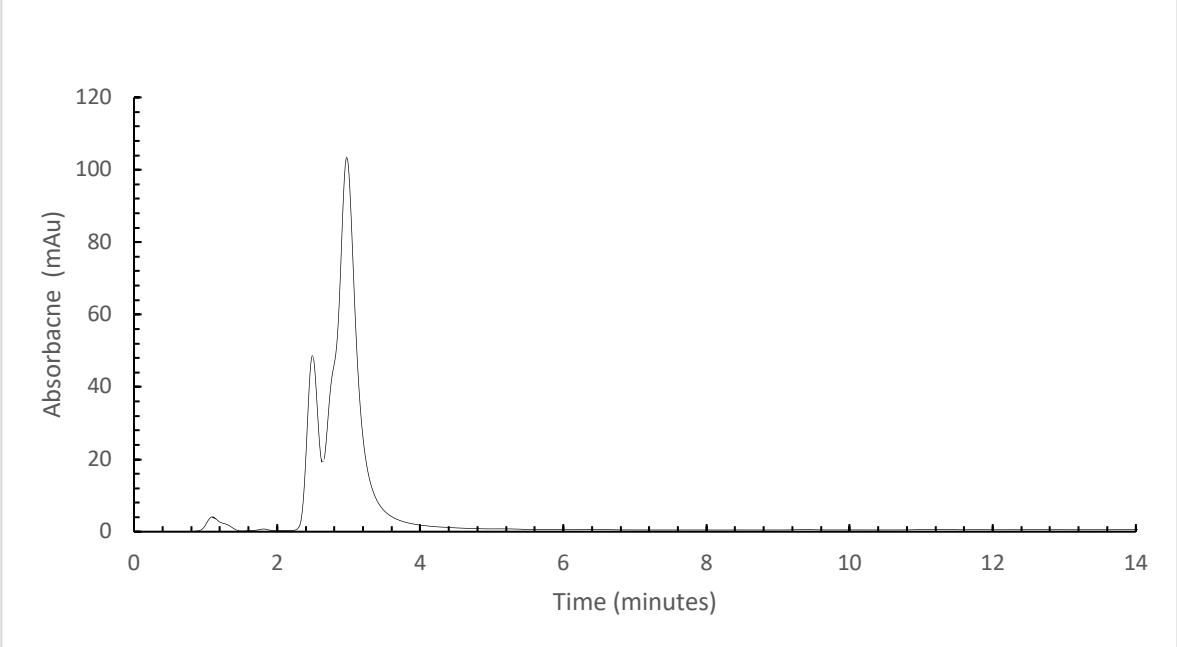

Figure 19: Condition D. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 2.496 and 2.974. This sample was analyzed with the Thermoscientific: BDS Hypersil column.

Starting with condition C, the mixed illicit drug sample seemed to have complete overlap between all the compounds, and they all eluted extremely early giving a retention time of 1.765. Due to the overlap and inability to identify the different compounds, 60:40 was the next attempt to observe the separation. Condition D, shown in Figure 19, was a progression in the right direction. The peaks eluted at delayed time compared to the previous 50:50 ratio as well as separated from a single peak into the start of two individual peaks. The prediction was that if the percentage of bottle A increased, containing just the pH-adjusted buffer, the compounds would continue to separate due to the decreased amount of butanol specifically in the water-rich environment. To observe if this prediction was correct, the next step was to try 70:30, condition E, shown in Figure 20. As predicted, the compounds were able to be separated enough to visibly see three peaks. It was not the ideal baseline separation, but being able separate these compounds slightly was positive progress. The last set of percentages attempted was 80:20, condition F, which can be seen in Figure 21. This was the most successful of all the trials, comparatively it was able to separate the compounds more than any other ratio, and it almost had baseline separation. Although much progress has been made, the peak shape and the lack of baseline separation need to be addressed before any method can be established.

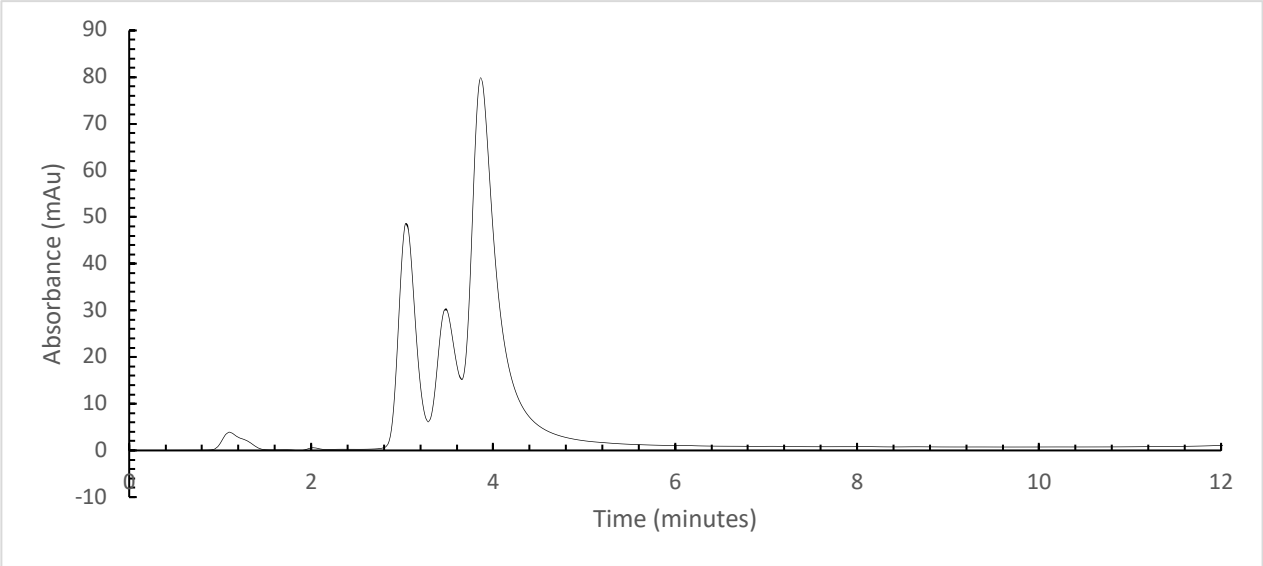

Figure 20: Condition E. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 3.047, 3.480, and 3.867 minutes, respectively. This sample was analyzed with the Thermoscientific: BDS Hypersil column.

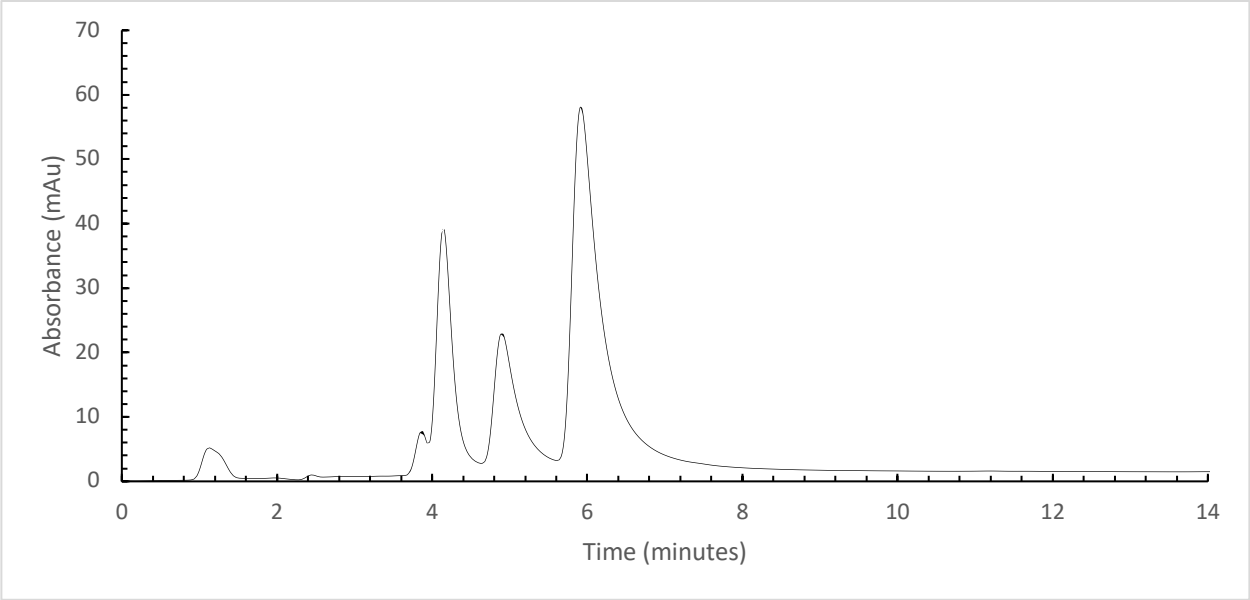

Figure 21: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 4.12, 4.921, and 5.907 minutes, respectively. This sample was analyzed with the Thermoscientific: BDS Hypersil column.

After these conditions and column could not progress the separation any further, another alteration needed to be made, and that change was in the form of a new column. The Thermoscientific: BDS Hypersil was changed to the Waters: Atlantis T3 column. There were three differences between them that could affect the compound separation, and those include the length of the column, the particle size, and the pore size. The column dimension increased in length, the pore size decreased but could be beneficial for these smaller molecules, and the particle size decreased. The particle size, when combined with the shorter column length, can produce fast high-resolution separation, which would be imperative for this research. [20] The conditions (F) remained the same, so the only thing altered was the type of column, this can be seen in Figure 22.

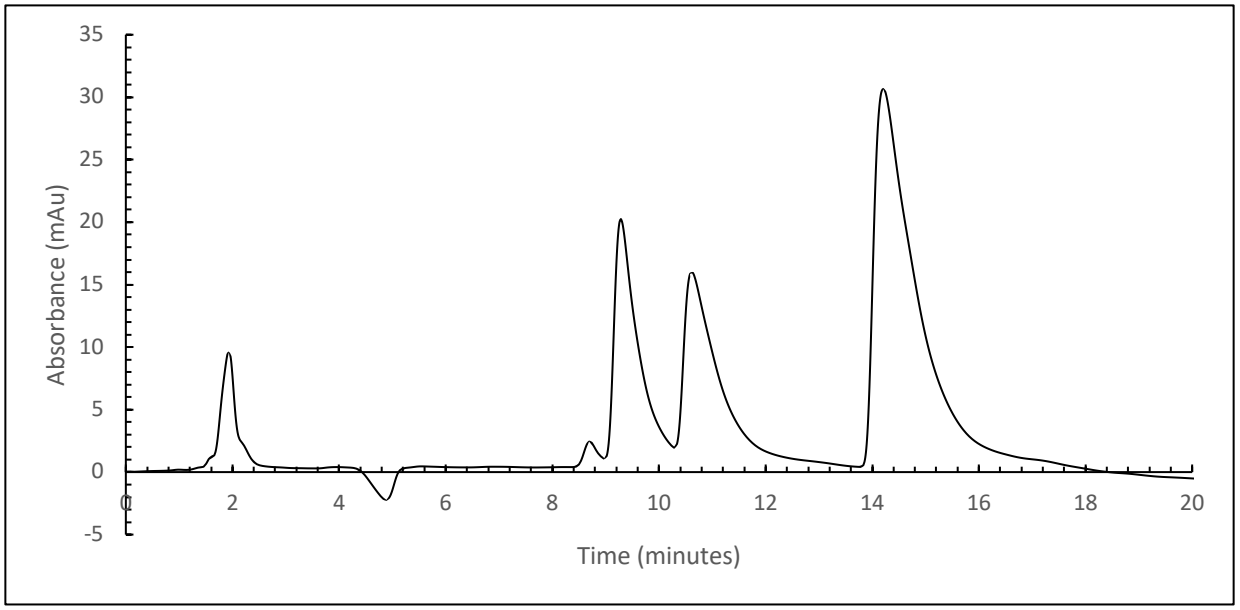

Figure 22: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 9.288,10.608, and 14.208 minutes, respectively. This sample was analyzed with the Waters: Atlantis T3 column at 25 ºC.

This change did have a positive effect on the separation of the compounds, there was almost complete separation between the methamphetamine peak and the MDEA peak, and the previous elution issue has been fixed since nothing is eluting before the solvent peak. Although, the peak shape is not ideal, they are very broad peaks, seem to have a bit of peak tailing, amphetamine has an unknown extra peak attached to it, and all that combine with the lack of separation between compounds suggests more research needed to be conducted. Previous research suggested altering the temperature could fix the peak tailing that is occurring. The research suggests this could happen for a couple of reasons. It can reduce the column backpressure, the viscosity of the solvent determines the amount of pressure, so the idea is that as the temperature increases, the viscosity and the backpressure will decrease allowing the solvent to flow more freely. [21] Additionally, the higher temperature allows for a quicker exchange of ions between the mobile phase and stationary phase resulting in shorter retention times. [21] The current method has been utilizing a column temperature of about 25 ºC, so by increasing the temperature to 40 ºC it could decrease the amount of tailing. This alteration was made to the method and can be seen in Figure 23.

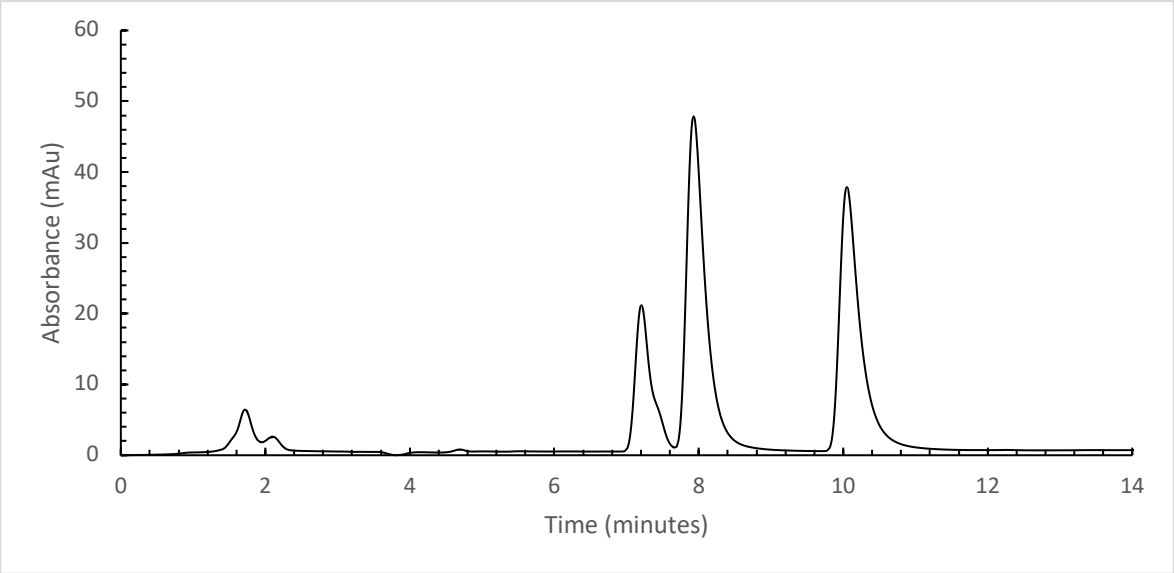

Figure 23: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 7.208, 7.932, and 10.053 minutes, respectively. This sample was analyzed with the Waters: Atlantis T3 column at 40 ºC.

The change in temperature had a significant impact on the sample, it allowed the for the methamphetamine and MDEA to be completely baseline separated, and the amphetamine and methamphetamine peaks are almost fully separated as well. The overall peak shape has a slight bit of tailing that was not corrected with the temperature adjustment, but a positive improvement from the previous trial. Additionally, the amphetamine peak had a slight shoulder, which does not match the gaussian template, but it does not discredit the resolution. The resolution of these peaks was all greater than 1.5, which is the U.S. Pharmacopeia standard for HPLC drug resolution. [17] Although, it is predicated that the misshapen peaks can be attributed to the silanol effect occurring in the column. Naturally, as the amount of butanol decreases the mobile phase should become less polar, therefore, the compounds elute quicker; the silanol effects have had the opposite effect, as the amount of butanol is decreased, the amount of separation increased. Although the exact mechanism of why this occurs is not known, it is predicted

that in basic compounds there are additional interactions. Most likely with the nitrogen and the hydroxyl groups in the analyte and the silanol in silica-based columns interact, therefore, causing an ion exchange between the protonated base and the salt. [15] These increased number of acidic silanol interactions can produce delayed retention and peak tailing. Some recommendations to avoid these interactions are having a higher buffer concentration (>10 mM) as well as adding a small amount of hexanenitrile which could potentially be used to cap or block a number of non-bonded surface silanol groups. [15,19]

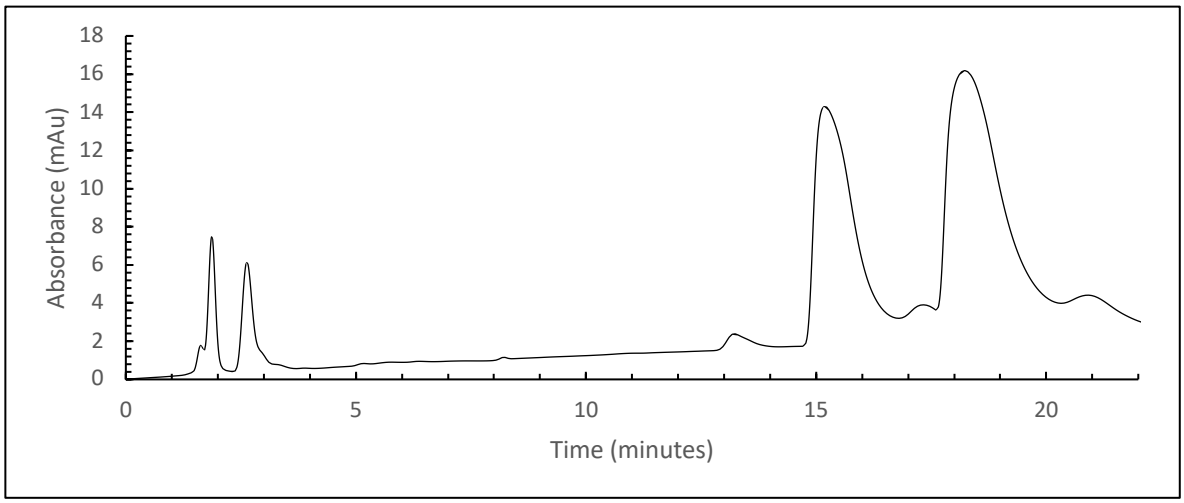

Figure 24: Condition F with 0.05% hexanenitrile. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 7.208, 7.932, and 10.053 minutes, respectively. This sample was analyzed with the Waters: Atlantis T3 column at 40 °C.

Adding the recommended 0.05% of hexanenitrile to the mobile phases was attempted, but the data was lacking as it did not have any benefit to the separation of the peaks or the peak shape. It is predicted that hexanenitrile masked the silica column preventing the butanol and compounds from interacting with the stationary phase, but the real reason is unclear.

With this newly established method, it was necessary to do a full comparison between the columns used during these experiments and observe if the results reflect the data recorded throughout this process. The Alltech: Prevail and the Thermoscientific: BDS Hypersil were analyzed using this method to identify the effect of temperature (Table 3). The chromatograms can be seen in Figures 25-28. As a tool for comparison, the Waters column was able to give the most separation between the illicit compounds and seemed to have the least amount of silanol activity. The other two columns produced data that seemed to have challenges separating as well as maintaining peak shape and resolution. Where some separation was achieved and the three compounds were visible, the baseline separation was not achieved, and were eluting early compared to the Waters column. It was concluded that changing column oven temperature has no effects on separation of compounds with Thermoscientific BDS hypersil and Alltech: Prevail columns.

Table 4: Column Comparison Conditions

Column	Mobile Phase A	Mobile Phase B	pH	Run Time (minutes)	Temperature (°C)
Alltech: Prevail	Phosphate Buffer	Phosphate Buffer with 3.5% butanol	7.0	25	25
					40
Thermoscientific: BDS Hypersil					25
					40
Waters: Atlantis T3					25
					40

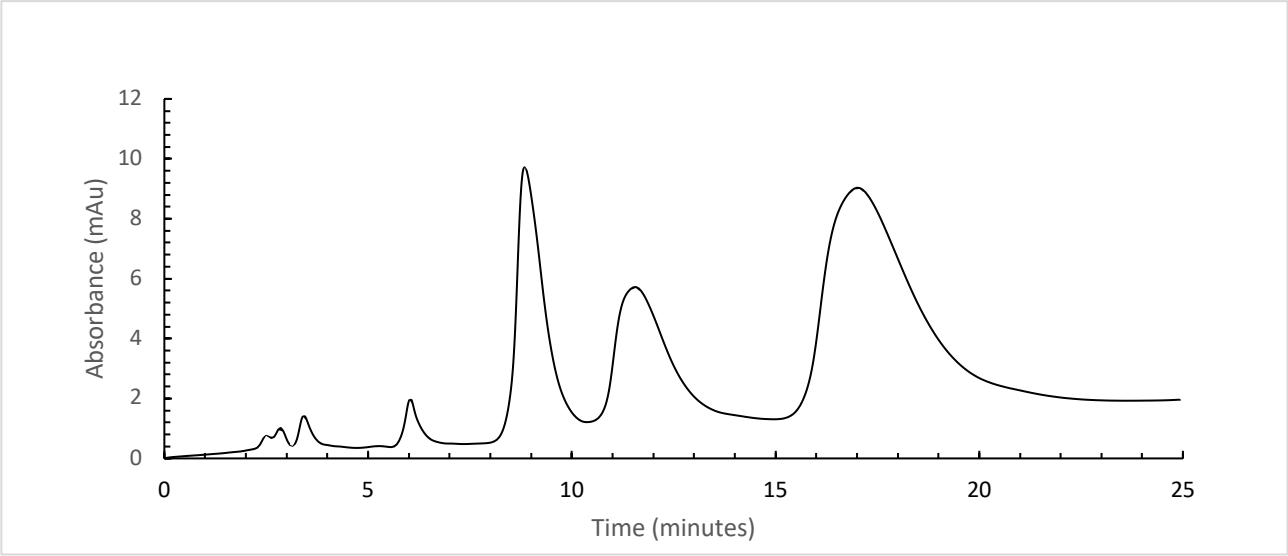

Figure 25: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 8.847, 11.556, and 17.017 minutes, respectively. This sample was analyzed with the Alltech: Prevail column at 25 ºC.

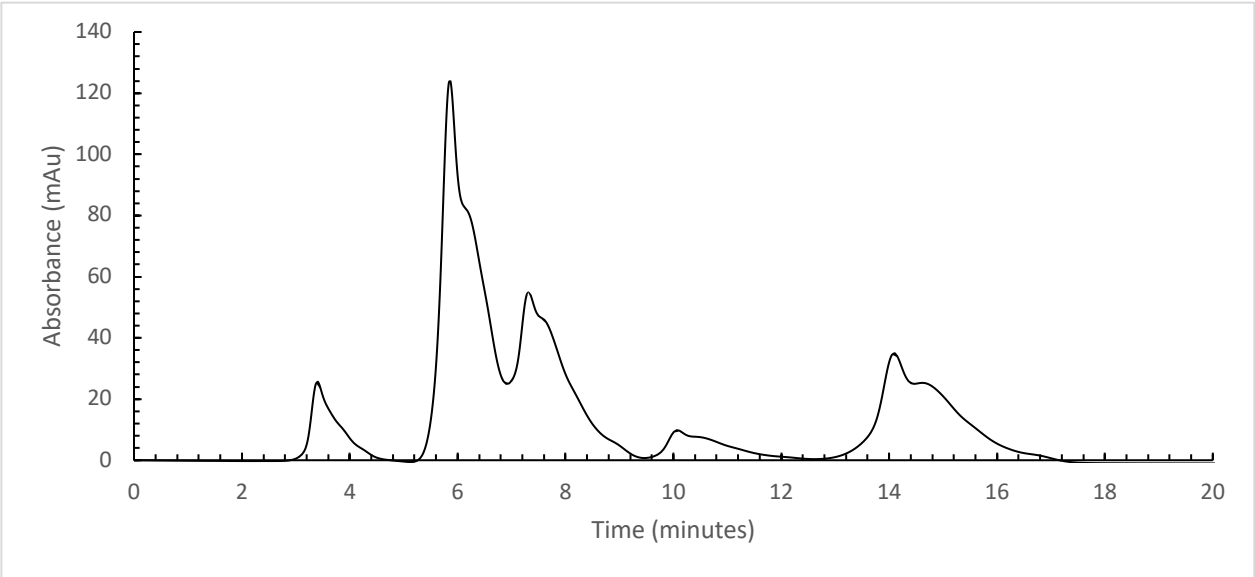

Figure 26: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA. The retention times of the sample could not be determined. This sample was analyzed with the Alltech: Prevail column at 40 ºC.

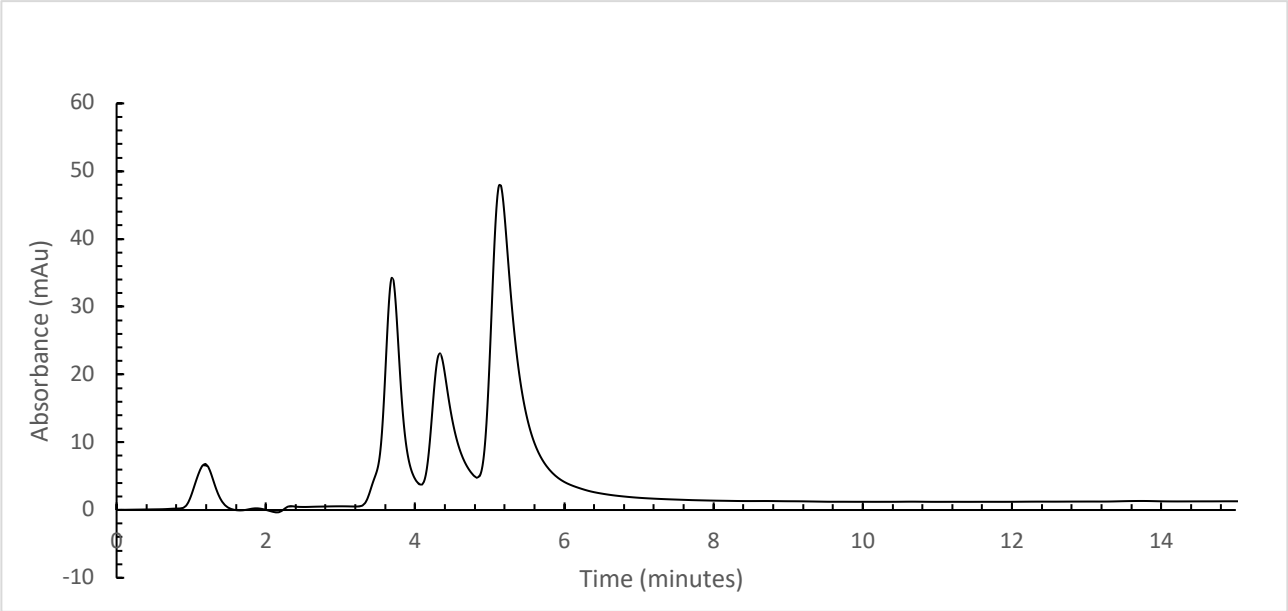

Figure 27: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 3.695, 4.334, and 5.140 minutes, respectively. This sample was analyzed with the Thermoscientific: BDS Hypersil column at 25 ºC.

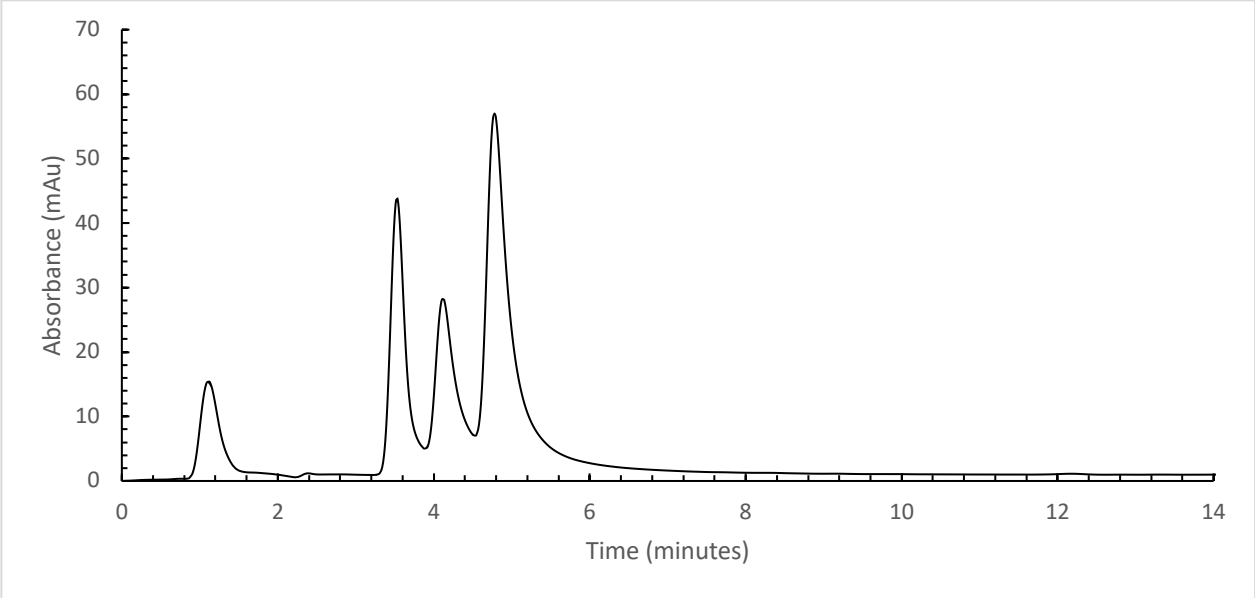

Figure 28: Condition F. This mixed sample included amphetamine, methamphetamine, and MDEA, and the retention times of the compounds are 3.529, 4.120, and 4.780 minutes, respectively. This sample was analyzed with the Thermoscientific: BDS Hypersil column at 40 ºC.

CHAPTER SIX - CONCLUSIONS

Based off the research conducted, the Waters column in conjunction with this water-rich mobile phase will be able to separate amphetamine-related compounds. The peak shapes did not achieve the ideal gaussian curve but did meet the resolution requirements. In the future, the peak shape can be altered by fully obtaining gaussian curvature, and this could be achieved by optimizing pH, butanol concentration and column oven temperature. This research did not observe any adverse effects on the column or the reproducibility of this method. The long-term effect on the degradation of the stationary phase in the column with a water-based mobile phase is unclear. Additionally, no other organic modifiers were analyzed due to the success of the 1-butanol during this research. In conclusion, this research set the foundation for illicit drug analysis utilizing a water-rich RP-HPLC mobile phases, this method could grow to be adapted for other drug classes and become an essential part of crime laboratory protocol.

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30. National Center for Biotechnology Information (**2023**). PubChem Compound Summary for CID 1183, Quinine.
31. National Center for Biotechnology Information (**2023**). PubChem Compound Summary for CID 1183, Acetylsalicylic acid.
32. National Center for Biotechnology Information (**2023**). PubChem Compound Summary for CID 1183, Amphetamine.
33. National Center for Biotechnology Information (**2023**). PubChem Compound Summary for CID 1183, Methamphetamine.
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