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Nicotine and what else?: HPLC elution optimization for the analysis of alkaloids found in electronic cigarettes

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Nicotine and What Else?:
HPLC Elution Optimization for the Analysis of Alkaloids Found in Electronic Cigarettes

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ABSTRACT

Electronic cigarettes, or e-cigarettes, have been scrutinized by the Food and Drug Administration and other public health organizations for issues related to quality control and efficacy. Erratic nicotine delivery and numerous toxic compounds in refill solutions have been reported. The ultimate goal of this research is to add to the growing amount of knowledge regarding e-cigarette ingredient content, safety, and quality. Standards of the tobacco alkaloids nicotine, anabasine, cotinine, and myosmine were prepared and quantified using high performance liquid chromatography (HPLC). Various elution schemes were tested and adjusted for optimal analyte resolution, and a final elution scheme was developed to characterize e-cigarette alkaloids.

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INTRODUCTION

Purpose

Due to the small number of studies regarding the safety and efficacy of electronic cigarettes, full ingredient content and concentrations remain uncertain in many varieties of electronic cigarette refill solutions. These products have only existed for sale in the U.S. for a relatively short time; very little comprehensive research has been conducted regarding their long-term health effects or associated risks. Further investigation is needed to address possible inconsistencies in the labeling of e-cigarette refill solutions. The objectives for this project are:

- to study the alkaloids found in electronic cigarettes,
- to study mobile phase theory as it pertains to the elution of nicotine and related alkaloids using high performance liquid chromatography (HPLC), and
- to develop an elution scheme capable of quantifying the alkaloids nicotine, anabasine, cotinine, and myosmine found in electronic cigarette filling solutions with HPLC.

What's an E-Cigarette?

Electronic cigarettes, also known as electronic nicotine delivery systems (ENDS), e-cigarettes, or e-cigs, are devices designed to deliver nicotine in a manner that mimics traditional tobacco smoking. Most e-cigarettes consist of a mouthpiece, a cartridge containing nicotine in solution, a heating element, an airflow sensor, a rechargeable lithium ion battery, and an LED. Upon inhalation, the sensor is triggered by negative pressure, activating the heating element which vaporizes the liquid

contained in the cartridge and heats air as it is inhaled. The LED located at the tip also lights up to imitate the burning of a traditional cigarette. The resulting aerosol made from the vaporized liquid and water vapor from the air creates an observable fog that is released upon exhalation.[1] Designs may vary according to brand; some e-cigarettes contained a fused cartridge and heating element called a "cartomizer" that is meant to be disposable. A diagram showing the inner components of a typical electronic cigarette is given in Figure 1.

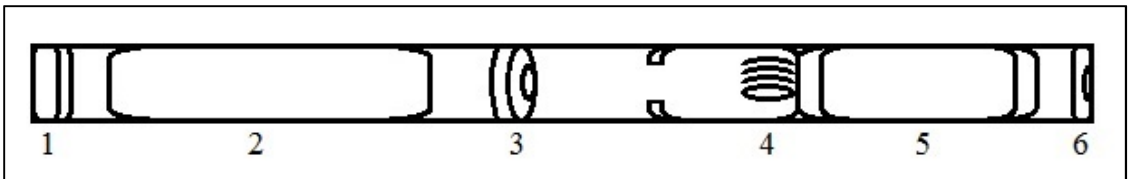

Figure 1: Electronic Cigarette Diagram. 1 = light-emitting diode (LED); 2 = lithium ion rechargeable battery; 3 = airflow sensor; 4 = heating element; 5 = filling solution cartridge; 6 = mouthpiece.

E-Cigarettes Vs. Traditional Cigarettes

While e-cigarettes superficially resemble traditional tobacco cigarettes, they are more closely related in internal design and operation to drug delivery systems such as inhalers and nebulizers.[1] The method of release for conventional cigarettes versus e-cigarettes is quite different: unlike conventional cigarettes, which release compounds in the form of smoke from combustion of tobacco, e-cigarettes release compounds in vapor form due to evaporation; for this reason, the term 'vaping' has been made analogous to smoking by e-cigarette industries and users alike.[2]

Once used, empty e-cigarette cartridges may either be disposed or refilled with liquid purchased from the manufacturer. These liquids contain nicotine marketed in a variety of concentrations, as well as food-grade flavorings and a humectant (typically propylene glycol and/or vegetable glycerin) with some variability depending on manufacturer and flavor of liquid purchased. Other ingredients may include ethanol along with other complex alcohols, diols, nicotine-related alkaloids and colorings.[3] Ethanol is often present as a flavoring component, or may be related to nicotine extraction from tobacco leaves.[4] Refill solutions come in a variety of flavors such as waffles, whiskey, pina colada, blueberry, and popcorn. Flavors other than tobacco and menthol are banned for conventional cigarettes under the Family Smoking Prevention and Tobacco Control Act of 2009 to prevent targeting tobacco products to youth; this ruling does not apply to any other tobacco products.[5] Other detected ingredients have included herbal preparations designed to have a therapeutic effect, or medications such as rimonabant and amino-tadalafil.[6] Some e-cigarette consumers even choose to prepare their own refill solutions by extracting nicotine from used tobacco cigarettes; a variety of homemade formulations containing ingredients similar to those listed above may be found online through electronic cigarette forums.

Production & Sales of E-Cigarettes

E-cigarette technology was first patented in 2003 by Hon Lik, a Chinese pharmacist who developed the atomization device that vaporizes nicotine in electronic cigarettes, cigars, and pipes.[7-8] The products were first marketed in China

by the company Ruyan (meaning 'like smoke') in 2004. [7,9-10] Ruyan received an international patent in 2007[7], and the e-cigarette has since been marketed worldwide in countries such as Brazil, Canada, Finland, Israel, Lebanon, the Netherlands, Sweden, Turkey, the United Kingdom, and the United States.[8,11]

Though mechanical electronic cigarette components such as the sensor, heating element, and cartridge are manufactured predominantly in China[7], refill fluids are produced domestically. One popular manufacturer produces refill liquids in Wisconsin for Blu, a company which maintains a significant portion of the American e-cigarette market.[12] Nicotine strength of refill solutions and cartridges vary from brand to brand in terms of reported equivalency to traditional cigarettes. Veppo states that one 10 mL bottle of refill fluid used over time is roughly equal to smoking ten packs of cigarettes[13]. In contrast, Vapor4Life equates 30 mL of its refill fluid to 25-30 replacement cartridges, each of which corresponds to approximately three-fourths to one whole pack of cigarettes. A single 30 mL bottle from Vapor4Life would therefore be comparable to smoking roughly 24 packs of cigarettes.[14]

E-cigarettes represent a rapidly growing industry. The market has doubled in size every year since 2008, and was projected to reach one billion dollars in sales in 2013.[15] The majority of electronic cigarette sales are from online, with a portion of sales attributed to mall kiosks and convenience stores where they may be sold without a tobacco license in certain parts of the U.S.[7, 16-17] In a study designed to collect information regarding e-cigarette product preferences and opinions, those who responded to surveys online were more likely to be aware of electronic cigarettes.[17]

Internet sales of electronic cigarettes have generated concern regarding their availability to minors who could not otherwise purchase tobacco or other nicotine delivery products. One study found that awareness of electronic cigarettes was inversely related to age, suggesting that young adults in the age range of 18-29 are most likely to try e-cigarettes.[18] Similarly, the awareness of e-cigarettes has been found highest among white, high-income, educated males from the ages of 18-24 who were self-reported nondaily smokers.[17] A study which compared the volume of internet searches for terms related to e-cigarettes with those of approved nicotine replacement methods found that e-cigarette searches first exceeded those of FDA-approved nicotine replacement therapy in 2008, and has since continued to rapidly outpace other forms of nicotine replacement.[19]

Advertising campaigns for electronic cigarettes resemble those of traditional cigarettes before they were banned in the U.S.—physically attractive men and women are shown vaping, with slogans such as "Cigarettes, You've Met Your Match" and "Rise from the Ashes".[20-21] Celebrity endorsements are also becoming increasingly prominent.[7] Commercials have aired on popular cable stations in the U.S., though broadcast channels have refrained from airing e-cigarette ads.[21]

The cost of e-cigarettes has decreased dramatically in conjunction with their rising popularity in the U.S.—companies which once sold units for as much as $200 in 2009 were as little as $21 as of 2012.[22] The devices are often advertised as cost-effective alternatives to traditional cigarettes. One survey found that users spent $33 per month for e-cigarette supplies as compared to an average of $150 to $200 per

month to smoke one pack of traditional cigarettes per day. The study suggests that cost is a major contributing factor to the popularity of the e-cigarette.[23]

Public Opinion & Regulation

A multitude of surveys have been performed and published regarding the general opinions of e-cigarette users with regards to perceived health benefits, awareness, satisfaction, and user demographics.[16-19, 23-31] Popular cited reasons for using e-cigarettes include a desire to quit smoking, reduced cost, the perceived ability to vape in places where smoking is normally prohibited, to avoid disturbing others while smoking, and a perceived reduction in health risks when compared to traditional cigarettes.[16] A majority of e-cigarette users reported in one survey that the product helped them quit or cut down on smoking and that e-cigarettes "feel healthier than smoking". Only about a third of respondents had tried to reduce e-cigarette consumption, however, and a majority of those who made attempts were self-reported as "not very successful".[24] These results suggest that while e-cigarettes may have the potential to reduce cigarette use among current smokers, they may not be effective at reducing nicotine dependence and carry a risk for abuse.

Despite their ubiquity in the U.S. market, formal regulatory classification for the e-cigarette is still under consideration. In 2008, the World Health Organization issued a warning to e-cigarette manufacturers stating that e-cigarettes should not be marketed as a smoking cessation tool. "WHO knows of no evidentiary basis for the marketers' claim that the electronic cigarette helps people quit smoking. Indeed, as far

as WHO is aware, no rigorous, peer-reviewed studies have been conducted showing that the electronic cigarette is a safe and effective nicotine replacement therapy".[11]

The American e-cigarette regulation dispute began when the U.S. Food and Drug Administration first attempted to halt Smoking Everywhere e-cigarette imports from China in 2008 on the grounds that the products were unapproved drug delivery systems under Federal Food, Drug, and Cosmetic Act. A U.S. District Court in Washington D.C. overruled the FDA's attempt to regulate e-cigarettes as drug delivery devices in 2010 and instead suggested that they fall under the regulatory description of tobacco products, defined as "any product made or derived from tobacco that is intended for human consumption".[32-35] This ruling directed the FDA to regulate e-cigarettes under the Family Smoking Prevention and Tobacco Control Act signed under the Obama administration in 2009. FDA representatives have stated that they would prefer regulating the products as drug delivery devices as it would offer greater control, allowing the administration to fully evaluate each electronic cigarette and refill solution for consumer safety and efficacy prior to sale.[33] While this recent ruling does not grant the FDA the power to block e-cigarette imports, it does grant the administration some control over marketing and quality control practices. Available options for the FDA to regulate e-cigarettes include removal from the consumer market for a premarket review, higher taxation, limitation of different flavors in tobacco products other than cigarettes, the introduction and/or expansion of warning labels, and restriction of manufacturer health claims.[5]

Other countries are currently evaluating similar approaches to the regulation of electronic cigarettes. The United Kingdom's Medicines and Healthcare Products Regulatory Agency cited an FDA study which found carcinogenic nitrosamines and diethylene glycol in refill solutions in its decision to try regulating e-cigarettes as medical products. This form of regulation would result in a similar testing and approval process prior to allowing the sale of different electronic cigarettes in the U.K.[36]

E-Cigarette Alkaloids: General Properties

Existing patents for e-cigarette refill formulations and the presence of tobacco-specific impurities suggest that the nicotine added to some refill solutions is not chemically pure but is rather an extract from tobacco. An alkaloid is generally defined as a naturally occurring, basic, nitrogen-containing compound that exhibits a pharmacological effect. Alkaloids can be found in food such as potatoes, tomatoes, coffee, tea, cocoa, and pepper.[37] Common tobacco alkaloids include: nicotine, cotinine, nicotine-1'-*N*-oxide, nornicotine, nicotyrine, nornicotyrine, myosmine, 2',3-bipyridyl, anabasine, and anatabine; over 20 pyridyl-type alkaloids have been found in tobacco.[37-38] Minor tobacco alkaloids have been found to amplify the physiological effects of nicotine.[39] Nicotine, anabasine, cotinine, and myosmine were chosen for this study both for their cost and in response to preexisting research conducted by the FDA which detected the presence of these alkaloids in e-cigarette refill solutions.[6] The tobacco alkaloids are all structurally based around pyridine and are substituted at the 3-position with an alicyclic base; nicotine, cotinine, and myosmine all contain a

modified pyrrolidine ring, whereas anabasine contains a piperidine ring. Most are weakly basic and thus have the capacity to ionize in an acidic solvent. The structures of tobacco alkaloids of interest and estimated pK_a values are listed in Figure 2. Tobacco alkaloids affect the body by binding to nicotinic acetylcholine receptors at the protonated alicyclic base; compounds such as cotinine whose pK_a values are below physiological pH are regarded as inactive under these conditions.[38]

Nicotine

Nicotine is the primary alkaloid found in tobacco (*Nicotiana tobacum*). While it is generally known as the addictive agent found in recreational tobacco products, it has been studied for its potential to treat Alzheimer's disease, Parkinson's disease, and ulcerative colitis.[40] (S)-(-)-nicotine predominates in its natural state and possesses biological activity, though the enantiomer (R)-(+)-nicotine may form due to added heat while smoking.[38, 40] Nicotine is regarded as one of the most heavily abused substances of all time—smoking is the top preventable cause of death in the world, and is estimated to cause one in every five U.S. deaths through both direct and indirect smoke exposure.[41-42] It behaves as a stimulant in the body, acting on nicotinic acetylcholine receptors in the brain and triggering the release of dopamine along with a variety of other neurotransmitters.[40] Nicotine increases the heart rate, metabolism, and memory, as well as suppressing appetite.[38, 43-44] Although nicotine is very toxic, nicotine poisoning from cigarettes is rare, and occurs most often from cutaneous exposure or ingestion of tobacco leaves or pesticides. The estimated lethal adult oral

dose of nicotine is between 40 and 60 mg, or approximately 0.6 to 0.9 mg/kg; the average tobacco cigarette contains 0.8 to 1.1 mg of nicotine.[38, 45]

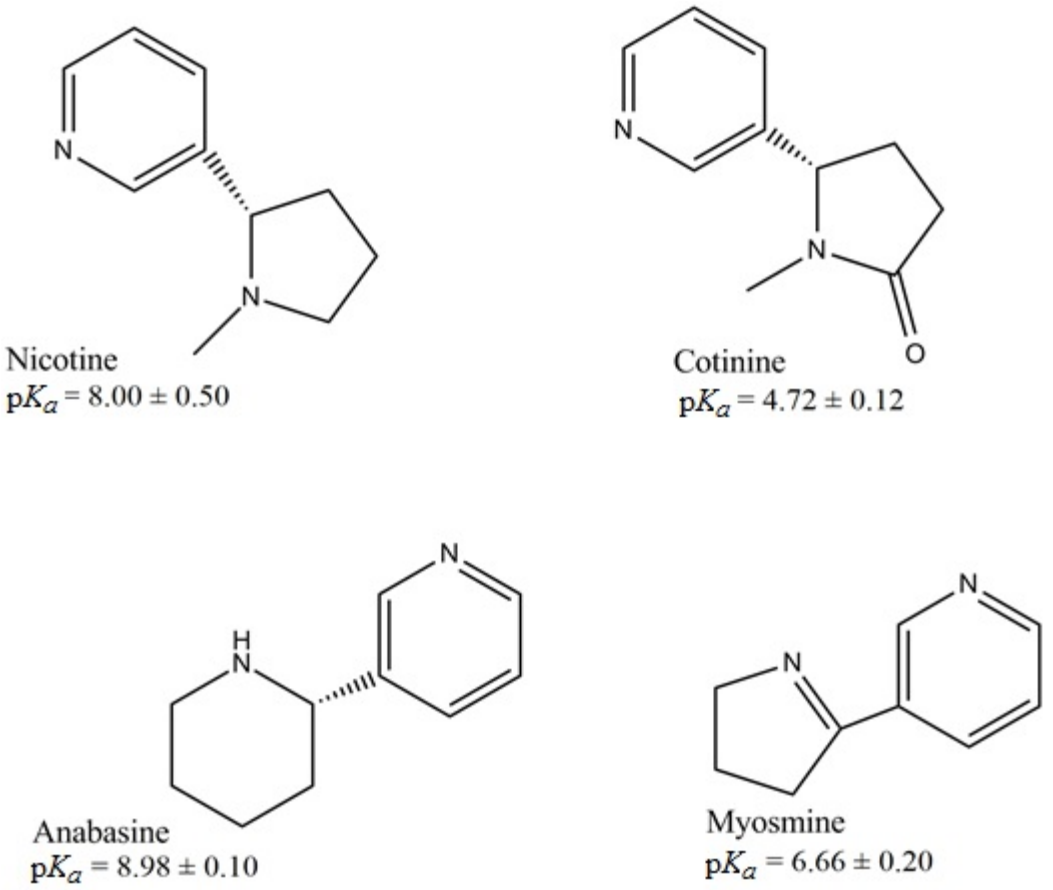

Figure 2: Structures of the Tobacco Alkaloids Nicotine, Anabasine, Cotinine, and Myosmine With Predicted pK_a Values[46]

Anabasine

Anabasine is perhaps best known for its historical use as an insecticide.[47] It is thought that nicotine alkaloids are produced by the tobacco plant as a natural insect repellant.[48] Anabasine may also be detected in cigarette smoke and body fluids of smokers. Due to its structural similarity, anabasine is thought to react similarly to

nicotine in the body. The median lethal dose for anabasine in mice is estimated between 11 and 16 mg/kg.[47] Anabasine and similar alkaloids containing piperidine are known to be teratogenic to livestock, resulting in defects such as cleft palate and multiple congenital contractures.[49]

Cotinine

Between 70 and 80% of nicotine in the body is oxidized in the liver with the addition of a carbonyl group on the pyrrolidine ring to form cotinine, a major tobacco alkaloid and nicotine metabolite, before undergoing further oxidation to other compounds.[50-51] The long half-life of cotinine (16 to 20 hours) compared to that of nicotine (1 to 4 hours) makes cotinine a good candidate for detection in analytical studies to determine a subject's nicotine intake.[52] Cotinine is suspected to have its own reactive mechanism distinct from nicotine in the body, though it has been shown to cause behavioral responses similar to that of nicotine.[53-54] It is less toxic than nicotine, and does not produce withdrawal symptoms.

Myosmine

Myosmine is found in very small quantities in tobacco; amounts found in cigarette smoke are 50 to 100 times less than nicotine.[55] It is genotoxic and has the potential to form carcinogenic intermediates such as *N*'-nitrosonornicotine in the body. Interestingly, myosmine has been detected not only in tobacco, but also in a variety of foods such as cereals, nuts, and milk.[56] Myosmine has been detected in non-smokers' plasma and saliva at levels as much as 5 ng/mL; these results indicate that myosmine is entering the body through additional routes besides passive

smoking.[55] The cutoff alkaloid concentration used to determine if a subject has been smoking is above 5 ng/mL. Tests measuring nicotine intake therefore remain valid even if myosmine is present in the body from sources unrelated to smoking.[57]

Existing E-Cigarette Research

Common e-cigarette refill solution ingredients and exhalation byproducts have been identified and quantified by both high performance and ultra-performance liquid chromatography, gas chromatography, and NMR spectroscopy.[2-3, 6, 58-62] Concentrations of ingredients vary widely according to the manufacturer of the refill solutions; for example, humectants may be found in a mixture or one may be omitted in favor of another, i.e. vegetable glycerin instead of propylene glycol or vice versa. Water content has also been found to vary from less than 1% to as much as 20%.[62]

Pre-existing research has indicated a need for further studies regarding the safety and efficacy of e-cigarettes. Quality control studies have found inconsistent or unclear labeling of nicotine and full ingredient content in refill solutions; nicotine content is often simply listed in milligrams, with no indication of whether this volume is per container or per milliliter.[9] As stated previously, the median lethal dose of nicotine is 40 to 60 mg.[38, 45] Several of the products among the samples tested claim to contain well above the lethal dose of nicotine in a single bottle; a 15-mL bottle with a reported strength of 36 mg/mL, for example, would contain as much as 540 mg of nicotine. A study which measured aerosol generation from e-cigarettes found that nicotine delivery and vapor production is often erratic between brands of the same strength and even from puff to puff of the same e-cigarette.[63] Studies measuring

carbon monoxide exhalation, heart rate, and/or nicotine levels in the blood plasma of e-cigarette users all demonstrate that nicotine delivery and absorption is less than that of conventional cigarettes, suggesting that e-cigarettes may be inefficient nicotine delivery devices.[64-65] Other research indicated that nicotine is present in e-cigarette vapor, presenting a secondhand risk of absorbing alkaloids that is currently not addressed by e-cigarette manufacturers.[2] Nicotine related impurities have been found in refill solutions, including the toxin diethylene glycol, as well as carcinogenic nitrosamines that are formed from tobacco alkaloids.[60, 66] Furthermore, 'vaping' an e-cigarette has adverse short-term effects on the pulmonary system.[67] E-cigarette vapor is also reported to contain volatile organic compounds present in tobacco smoke; other vapor constituents include flavorings and glycerin.[2] Finally, since e-cigarettes have existed in the U.S. for less than 10 years, there have been no studies assessing the long-term health risks or effects associated with electronic cigarette use or direct exposure to refill solutions. The ultimate goal of this e-cigarette research is to add to the growing amount of knowledge regarding content and quality of filling solutions.

CHROMATOGRAPHIC THEORY

Liquid Chromatography

Liquid chromatography is a type of chemical separation that occurs between a liquid mobile phase (known as the eluent) and a solid stationary phase, or adsorbent, contained in a column. The sample containing the compound(s) of interest mixed with impurities, solvent(s), and/or unknown compounds is placed on the column via an injection port. While passing through the column, compounds in the sample separate from each other due to their chemical properties such as molecular weight, polarity, and pK_a. The sample then passes through a detector that displays each detectable compound in the sample as peaks on a chromatogram. Peaks may be interpreted by their retention times and area—each compound possesses a unique retention time, and peak area is related to the amount of each compound. A diagram depicting a typical HPLC apparatus is given in Figure 3.

High performance liquid chromatography, or HPLC, is a common separation method used in analytical chemistry. It is often chosen for its sensitivity, adaptability to both identification and quantification, and for its ability to separate a variety of organic, inorganic, and organometallic compounds.[68] It is able to detect micrograms of a sample without the decomposition of analytes that is encountered in gas chromatography.[69] HPLC uses pressures of up to 6000 psi mediated by a reciprocating pump to push liquids through the column; the pressure is often maintained using a pulse dampener, which allows for reproducibility and fine control of the eluent flow rate, typically between 0.1 and 10 mL/min.[68] Samples ranging

between less than 1µL and 500 µL in volume are introduced to the column at high pressures using a sampling loop. Columns contain a tightly packed adsorbent designed to slow down the sample in the mobile phase for separation. Common column packing materials range from 3 to 10 micrometers in diameter. In reversed phase liquid chromatography (RP-HPLC), a polar solvent such as water or acetonitrile is pushed through a nonpolar column material such as organically-modified silica. Reversed-phase chromatography is vastly preferred over other forms of HPLC: almost 90% of all liquid chromatographic analyses feature RP-HPLC. The main reasons in favor of RP-HPLC are the ability to distinguish between very closely related compounds and the extent to which the mobile phase may be manipulated to optimize the elution profile.[70] RP-HPLC was therefore chosen as the instrumental method for this study.

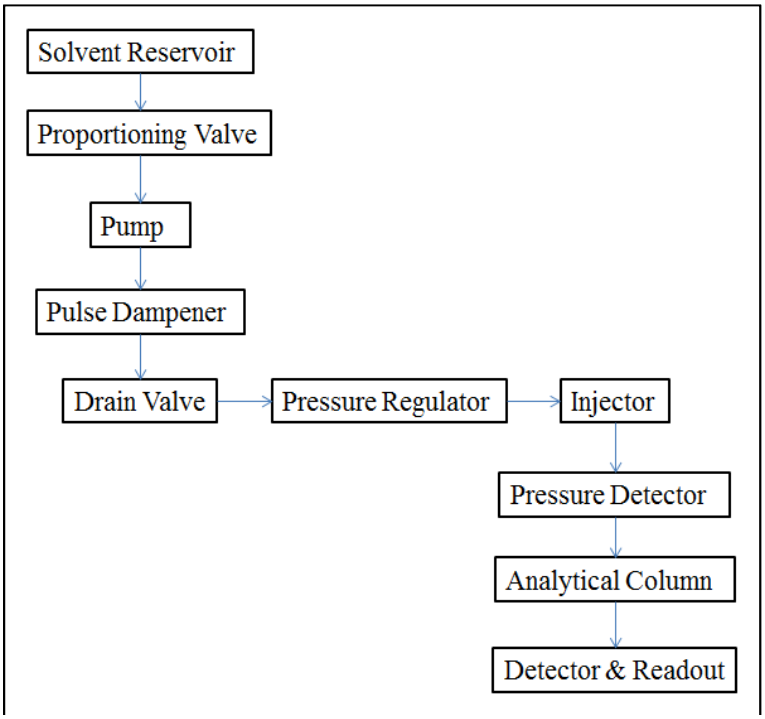

Figure 3: Block Diagram of HPLC Instrument Components

Compound Identification & Detectors

Identification of compounds in a sample may be achieved through a combination of their retention times and detector output. The UV absorbance and retention of the compounds were used for identification in this experiment. The retention time is defined as the time measured from the initial injection of sample to the peak absorption time. The void time, or T_M, is the time required for unretained compounds to pass through the column and reach the detector following injection. Retention times should be adjusted such that analytes do not co-elute with the small peak corresponding to T_M. A Thermo Finnigan chromatograph was used for preliminary analysis and featured a UV detector with a deuterium lamp wherein the displayed amount of absorbed ultraviolet light is related to the amount of compound passing through the detector. The wavelength of maximum absorbance, or λ_{max}, is unique to the compound being studied. The λ_{max} may be estimated by identifying different functional groups known as chromophores in the compound that absorb ultraviolet light. Depending on the detector model, the wavelength of detection may be adjusted on the HPLC according to the λ_{max} of the organic compound of interest. Diode array detectors such as the one found in the Agilent 1220 chromatograph are capable of simultaneous detection at multiple wavelengths, allowing the experimenter to study mixed samples containing compounds with different λ_{max} values. The λ_{max} is generally chosen as the wavelength of detection for a compound because it yields the strongest signal, resulting in improved peak shape and a higher signal-to-noise ratio.[68]

The General Elution Problem

Due to the different chemical properties of compounds found in a mixed sample, achieving good resolution in conjunction with a reasonable retention time often proves to be difficult. A single set of conditions is rarely ideal for all components that one may find in a sample. This is commonly known as the general elution problem, and knowledge of the factors influencing separation is required to overcome this issue. Often, gradient elution schemes in which the proportions of solvents are changed with respect to time are used so that the appropriate conditions may be met to elute a particular component at the desired time. This method can be used to prevent overlapping peaks, broad peaks, and/or long run times.[68]

Mobile Phase Theory

The liquid mobile phase used in HPLC may be easily manipulated at relatively low cost through a variety of factors to optimize retention time and resolution. Mobile phase adjustment is therefore the primary tool for controlling the elution profile—eluent composition, organic solvent, pH, and the buffer may all be changed in creating a unique separation mode. The vast majority of mobile phases in RP-HPLC when added to a column are hydro-organic mixtures, or mixtures of an organic solvent and water. Mobile phases must be very pure (HPLC grade or filtered) to prevent the appearance of background signals or deposition of impurities on the column. Solubility, pH, stability, and polarity should be considered among other factors when choosing an appropriate mixture for the mobile phase.[70]

Choosing the appropriate organic solvent to be mixed with water as well as adjusting its concentration has a large impact on the mobile phase strength, i.e. the degree to which the mobile phase causes the sample components to elute. Depending on the solubility of the analyte in the organic component of the mobile phase, increasing the organic solvent concentration may greatly improve or reduce the quality of peaks.[70]

Methanol and acetonitrile are preferred organic solvents due to their miscibility in water, availability, and relatively low UV absorption cutoff (less than 205 nm). Of the organic solvents studied, acetonitrile is considered a stronger solvent than methanol, meaning that a smaller added percentage of acetonitrile would be needed to influence the retention of a compound than methanol in a hydro-organic mixture.[70]

When organic solvents are added to the mobile phase, a shift in pH is often observed, even in buffered systems. Leveling effects should be considered when working with very strong acids or bases mixed in water—bases stronger than OH^- or acids stronger than H_3O^+ will not remain stable in aqueous solutions.[71] Shifts in pH observed with the addition of organic solvents are commonly due to multiple factors that affect the interpretation of pH. When a nonaqueous solvent is added, the conventional definition of pH as it relates to hydrogen ion activity α no longer applies to the solution. Glass electrodes such as the one used in this study are designed to make measurements relative to a standard, aqueous buffer as shown in Equation 1

$$pH = pH_{st} + \frac{(E - E_{st})F}{RT \ln 10} \qquad \text{Eq. 1}$$

where pH_{st} is the pH of the standard buffer solution (this is often set manually while calibrating the pH electrode), E is the cell voltage of the sample solution, E_{st} is the cell voltage of the standard buffer solution, F is the Faraday constant, R is the gas constant, and T is the temperature.[72] When the pH is measured using a glass electrode, the voltages are entered into the above equation to receive a digitally displayed pH reading. In order for this equation to be accurate and report a pH value that corresponds to α, the solution must be dilute, aqueous, and in the mid-pH range.

An increase in pH is typically found when adding an organic solvent such as methanol to an aqueous buffer.[73] This was observed in our study when we were forced to pre-mix solvents due to pump failure. The factors leading to this increase are expounded below:

- pH shifts may be caused by a change in the junction potential between the outer glass electrode and the inner reference electrode in combination pH probes such as the one used in this study. When an organic solvent is added, the junction potentials between the reference and the sample are unequal, leading to errors in pH measurements.
- A change in the autoprotolysis constant K_w can lead to pH shifts in organic solvents. In water, this constant is equal to $[H^+][OH^-] \approx 10^{-14}$. In hydro-organic mixtures, however, this is shifted due to the presence of additional ions—in the case of added methanol, for example, the CH_3O^- ion is responsible for additional anions in equilibrium with the hydrogen ion, leading to an apparent increase in basicity.

- The conventional pH scale ranging from 0 to 14 is altered when organic solvents are added to aqueous solutions. This change is related to the concentration of organic solvent. In methanol-water mixtures, for example, the apparent pH shift due to this effect is less than 0.1 pH units when the ratio of methanol to water is 50/50; however, in 100% methanol, this effect contributes to greater than 2 units in the overall pH shift of the solution.
- Finally, the buffer pK_a is altered in organic solvents due to different abilities to dissolve hydrogen ions under these conditions (see Buffers in RP-HPLC).[72]

These factors have led scientists to adopt a naming convention regarding the solution in which a pH sensor is calibrated as it compares to the solution in which the pH is actually measured. The term ${}^{s}_{s}pH$ is used when the pH sensor is calibrated in the same hydro-organic mixture as the sample solvent, whereas ${}^{s}_{w}pH$ refers to the pH measured when the electrode was calibrated in an aqueous solution and the sample pH measurement was taken in a hydro-organic mixture.[74] In this study, the measured pH of mixed mobile phases refers to the ${}^{s}_{w}pH$.

Buffers in RP-HPLC

Even slight changes in the degree to which a compound exists in its ionized state can lead to profound changes in its selectivity on a chromatography column.[75] When compounds in a sample are capable of ionization, it is therefore important to include a buffer in the mobile phase to prevent erratic results due to pH shifts that could lead to different ionized species in the column. Due to the nature of the reversed-phase column, compounds buffered at ionized states tend to elute first in a

mixed sample followed by non-ionized components.[70] Considerations for buffers in HPLC studies include the buffer identity, the buffer pH, the buffering capacity, and the effects of dilution and/or mixing of buffers with other solvents.

The buffer capacity is an important factor to consider when selecting the appropriate buffer for HPLC analysis. Buffer capacity β is defined as a buffer's ability to resist a change in pH when incremental amounts of acid or base are added to the solution.[76] It may be described using Equation 2

$$\beta = \frac{dC_b}{d(pH)} = \frac{-dC_a}{d(pH)} \qquad \text{Eq. 2}$$

where dC_b is the change in concentration of base, dC_a is the change in concentration of acid, and dpH is the shift in pH.[77] This equation demonstrates that the value for buffer capacity remains positive regardless of the respective positive or negative pH shift experienced when base or acid is added. β is proportional to both the concentration of the buffer and to its pK_a . The buffering capacity is strongest when the pH of the buffer is equal to the pK_a ± 1; specifically, this is obtained at the pH corresponding to the buffer's titration midpoint where the concentration of acid is equal to that of the conjugate base, or vice versa. It is therefore recommended to select buffers with pK_a values close to the target pH. This phenomenon is shown with Equation 3, known as the Henderson-Hasselbalch equation

$$pH = pK_a + log\frac{[A^-]}{[HA]} \qquad \text{Eq. 3}$$

where [A^-] is the concentration of the conjugate base and [HA] is the concentration of the acidic species. As the ratio [A^-]/[HA] approaches unity, the term is simplified to zero and the pH is equal to the pK_a.[78]

Deviations from the buffer pK_a can have drastic effects on the buffer capacity: when a buffer's pH is one unit from the pK_a, the buffer capacity is reduced by one-third; however, when the pH is two units from the pK_a, the capacity is reduced by a factor of 25.[77] Shifts in buffer pK_a can occur for ionic buffer species due to deviations from pK_a value listed in most literature sources. This is because simple pK_a calculations made from the Henderson-Hasselbalch equation do not normally account for shifts ionic strength due to the addition of H^+ or OH^- ions. Anionic acids such as hydrogen phosphate therefore experience an unexpected shift in the optimal buffering pH as the concentration of buffer is increased.[79]

Despite this, the addition of a buffer with a pH within two units of the pK_a is often sufficient to separate compounds when using HPLC. This is because analytes are often present at very low concentrations once they are separated, making even low buffer capacities adequate for controlling analyte ionization.[76]

EXPERIMENTAL

Materials

Cotinine lot 10165472 and nicotine lot 10139194 were purchased from Alfa Aesar. Myosmine lot 072M4114V and anabasine lot MKBN2341V were purchased from Sigma Aldrich. The HPLC-grade eluents water, acetonitrile, and methanol were purchased from Fisher. Phosphate buffers were prepared using monobasic sodium

phosphate monohydrate and dibasic sodium phosphate purchased from J.T. Baker Chemical Company.

Standard Preparation

Nicotine, anabasine, cotinine, and myosmine were diluted with HPLC-grade water to concentrations of 1 mg/mL to make stock solutions of each alkaloid. These stock solutions were further diluted with HPLC-grade water to a range of concentrations appropriate for the HPLC. These concentrations were selected to fall within the reported concentration range of e-cigarette filling solutions when diluted by a factor of 1000. Common e-cigarette filling solutions allegedly range in strength from 0 to 36 mg/mL; the pure alkaloids were therefore chosen to be diluted to strengths of 5, 15, 25, and 40 µg/mL. This range allows the presumed concentrations of the filling solution samples to fall within the calibration curves for the standards. Once the alkaloids were successfully identified using HPLC, mixed samples containing all four alkaloids of the same concentration were similarly prepared with HPLC-grade water in order to develop an elution scheme that would successfully elute all four compounds.

Instrument Conditions

A Thermo Finnigan SpectraSystem chromatograph was used for preliminary determination of the elution scheme in this study. This chromatograph features a deuterium lamp for spectroscopic UV detection and a reversed phase Thermo Hypersil C-18 column with a manual injection system. The majority of attempted elution schemes were developed on this instrument.

An Agilent 1220 Infinity Series chromatograph was purchased during the course of this research. It includes a diode array detector capable of simultaneous detection at a variety of wavelengths, and a 15-cm long reversed phase Poroshell 120 C-18 column with attached amides.[80] This instrument and column allowed for improved reproducibility, better detection limits, and included an automated injection system. For both the Thermo Finnigan and Agilent instruments, injection volumes were 5 μL and the flow rate was set to 1.0 mL/min. A final successful elution scheme was developed on this instrument.

In order to ensure the presence and accuracy of the concentrations of prepared standards, ultraviolet-visible spectrometry (UV-vis) was used for each alkaloid concentration. A Varian Cary 100 Bio UV-vis spectrophotometer was used to scan the ultraviolet-visible spectrum for each compound, allowing for the determination of λ_{max} needed for detection using the chromatograph.

RESULTS & DISCUSSION

UV-Visible Spectra

Absorption spectra for the alkaloids are given in Figures 4-7. Analysis of the alkaloids using UV-vis absorption spectrometry found an expected λ_{max} value of 260 nm for nicotine, anabasine, and cotinine.[81] The displayed absorption spectra for some of the compounds indicate a negative absorption value for the lowest tested concentration (5 µg/mL); this is due to instrument drift.

Interestingly, myosmine had two distinct absorption peaks at both 234 nm and 260 nm. A shift in λ_{max} towards lower wavelengths (the blue end of the spectrum) is known as a hypsochromic shift.[68] The structure of myosmine reveals an additional double bond in the pyrrolidine ring (see Figure 2)—contrary to the observed results, the extended conjugation of the pyridine ring found in this molecule would typically indicate a red, or bathochromic, shift in absorption.[82] The unexpected additional peak at 234 nm is in fact thought to be due to a bathochromic shift in the absorption spectrum of the pyridine ring that would normally exist below 200 nm.[81]

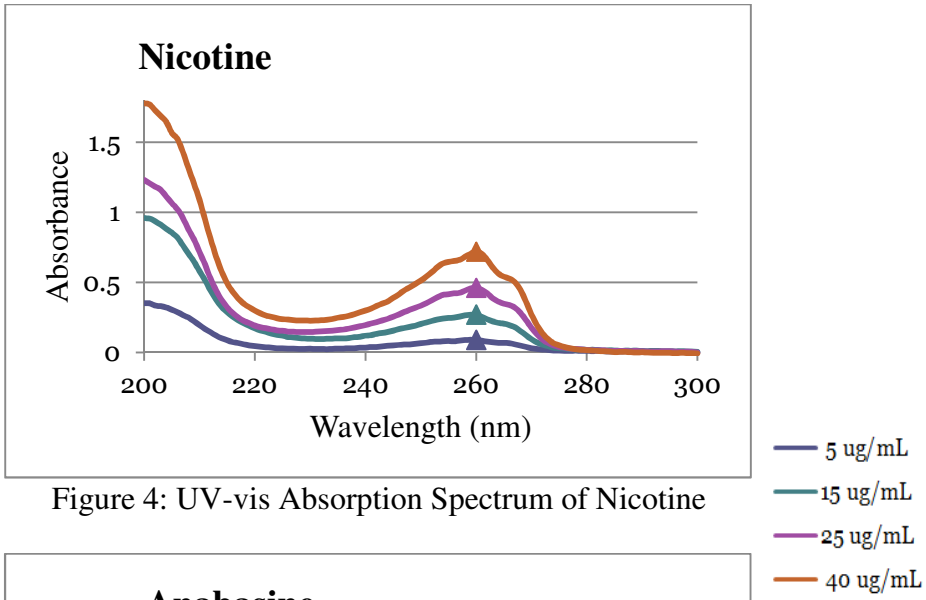

Figure 4: UV-vis Absorption Spectrum of Nicotine

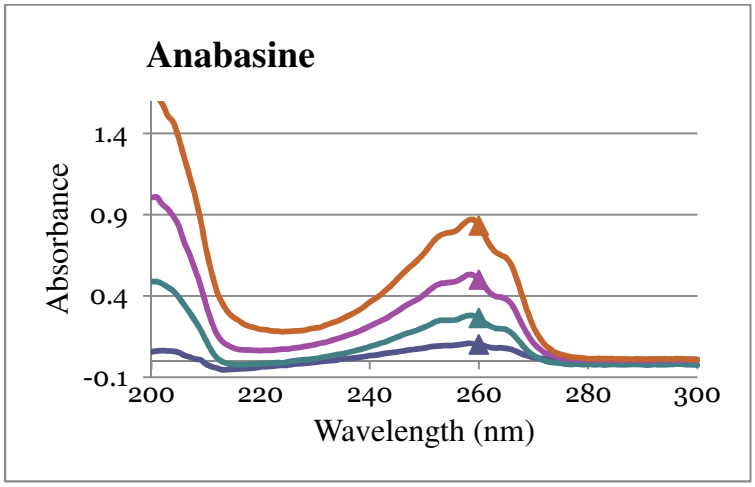

Figure 5: UV-vis Absorption Spectrum of Anabasine

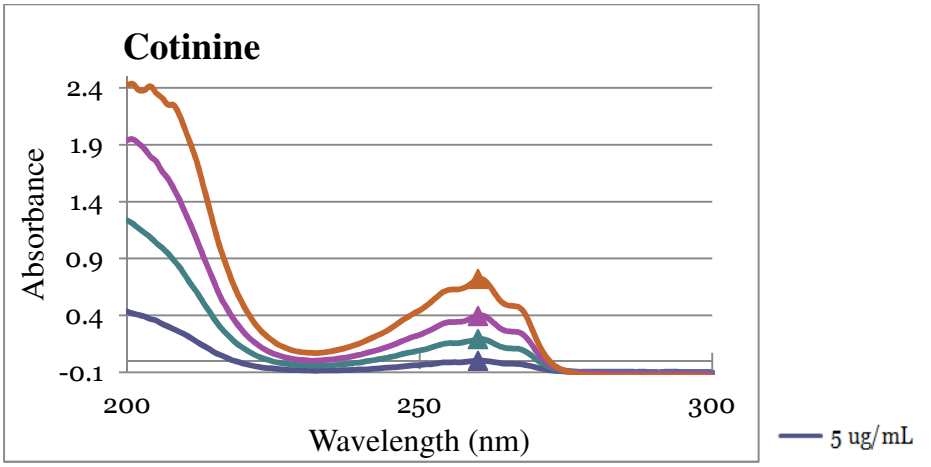

Figure 6: UV-vis Absorption Spectrum of Cotinine

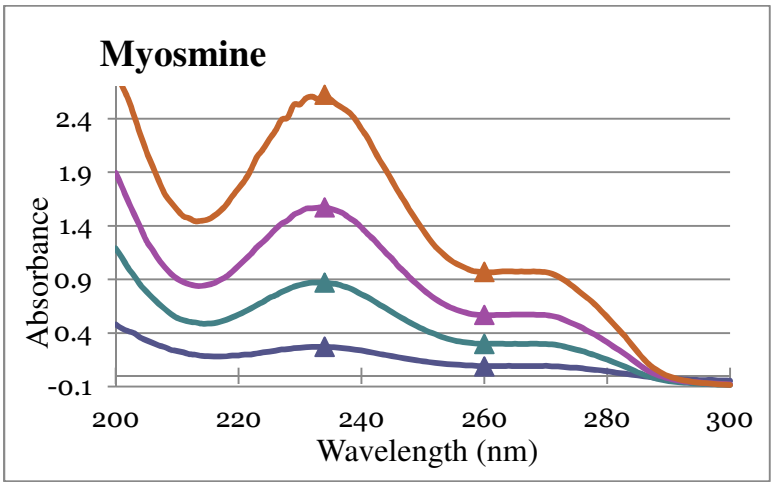

Figure 7: UV-vis Absorption Spectrum of Myosmine. Note the presence of two absorbance peaks located at 234 nm and 260 nm.

The existence of two absorbance peaks for myosmine proved to be a valuable tool for identification of this compound when studied as part of a mixed sample for HPLC. By using the Agilent instrument's diode array detector, the mixed sample could be injected once and reported using wavelengths of detection set to both 260 nm and 234 nm. This enabled myosmine to be quickly and easily differentiated from the other alkaloids present in a sample. This is shown in Figure 12.

Mobile Phase Development

During the course of this project, the conditions of the HPLC eluent such as buffer pH, buffer identity, solvent ratio, and solvent identity were adjusted in order to achieve well-defined peaks and/or good resolution in mixed samples. The various attempted mobile phases are given in Table 1.

The first attempted elution scheme for this project was taken from an HPLC analysis of e-cigarette alkaloids funded by the FDA. According to Table 1, eluent A consisted of 100% acetonitrile and eluent B consisted of 10% acetonitrile in 20 mM ammonium formate buffered to a pH of 8.7. This elution scheme featured a complex gradient that consisted of multiple adjustments over an extended period of time: from 0 to 10 minutes, the elution scheme was programmed to transition from 100% eluent B to 80% eluent B and 20% eluent A; from 10 to 20 minutes, the scheme transitioned to 10% eluent B and 90% eluent A; from 20 to 21 minutes, the scheme transitioned to 100% B; and from 21 to 30 minutes, the scheme remained constant at 100% B.[6]

One possible reason for the inability of this elution scheme to resolve the alkaloids in this study was the rapid transition from 20 to 21 minutes in the relative amount of eluent B—it is possible that this did not allow enough time for the column to equilibrate to eluent B before the next sample was introduced to the instrument. Another reason may be the condition of the Thermo Finnigan instrument at the time of the study. Variable pressure and issues regarding reproducibility of results were observed prior to the failure of one of the eluent pumps. This restricted the Thermo Finnigan instrument to the use of a single eluent.

Table 1: Attempted Elution Schemes						
	Eluent(s)		Method			
Mobile Phase No.	A	B	A/B	pH of Buffer	Gradient/ Isocratic	Instrument
1	ACN	10% ACN in 20 mM formate		8.7	Gradient (Trehy)	Thermo Finnigan
2	MeOH	HPLC H_2O	25/75	NA	Isocratic (Kubica)	Thermo Finnigan
3	MeOH	10 mM phosphate	65/35	6.8	Isocratic (Tambwekar)	Thermo Finnigan
4	MeOH	10 mM phosphate	80/20	6.8	Isocratic	Thermo Finnigan
5	MeOH	50 mM phosphate	80/20	7.9	Isocratic	Thermo Finnigan
6	MeOH	50 mM phosphate	65/35	8.2	Isocratic	Thermo Finnigan
7	ACN	none	NA	NA	Isocratic	Thermo Finnigan
8	ACN	50 mM phosphate	50/50	8.5	Isocratic	Thermo Finnigan
9	ACN	25 mM phosphate	80/20	4.0	Isocratic	Thermo Finnigan
10	ACN	25 mM phosphate	50/50	4.0	Isocratic	Thermo Finnigan
11	ACN	25 mM phosphate	35/65	4.0	Isocratic	Thermo Finnigan
12	ACN	25 mM phosphate	60/40	7.0	Isocratic	Thermo Finnigan
13	ACN	25 mM acetate	50/50	6.0	Isocratic	Thermo Finnigan
14	ACN	25 mM acetate	35/65	6.0	Isocratic	Thermo Finnigan
15	ACN	25 mM phosphate	35/65	7.9	Isocratic	Agilent
16	MeOH	25 mM phosphate	35/65	7.9	Isocratic	Agilent
17	MeOH	25 mM phosphate	20/80	7.9	Isocratic	Agilent
18	MeOH	25 mM phosphate	50/50	7.9	Isocratic	Agilent
19	MeOH	25 mM phosphate	35/65	7.9	Gradient*	Agilent

MeOH = methanol, ACN = acetonitrile. Elution schemes are listed in the order they were attempted. *Multiple adjustments were made to this gradient scheme.

As a result, many mixed hydro-organic eluents were prepared when attempting later elution schemes on this instrument by combining the buffer and the organic eluent prior to analysis.

With the development of new elution schemes, adjustments to the buffer pH, hydro-organic solvent ratio, and buffer concentration were made incrementally according to the observed retention characteristics of the alkaloids tested. The most successful elution scheme developed for the Thermo Finnigan HPLC is given below in Figures 8-11. This elution scheme consisted of 35% phosphate buffered at a pH of 6.8 and 65% methanol run isocratically for 6 minutes (see mobile phase number 3 in Table 1). This result led to the development of a final successful elution scheme with similar specifications using the Agilent HPLC.

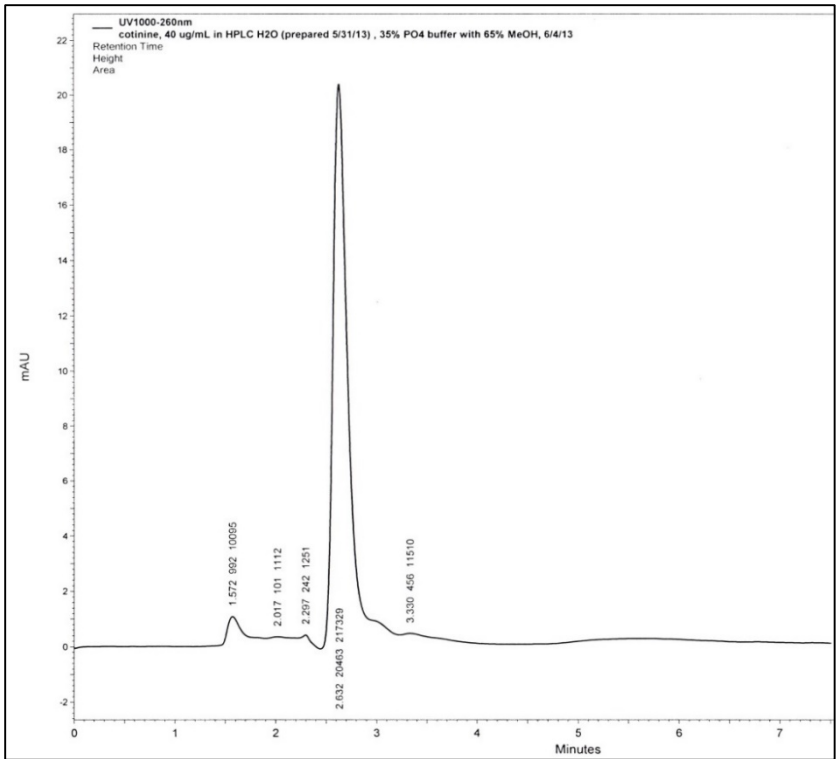

Figure 8: HPLC Chromatogram of Cotinine Using Thermo Finnigan HPLC.

Retention time = 2.632 min.

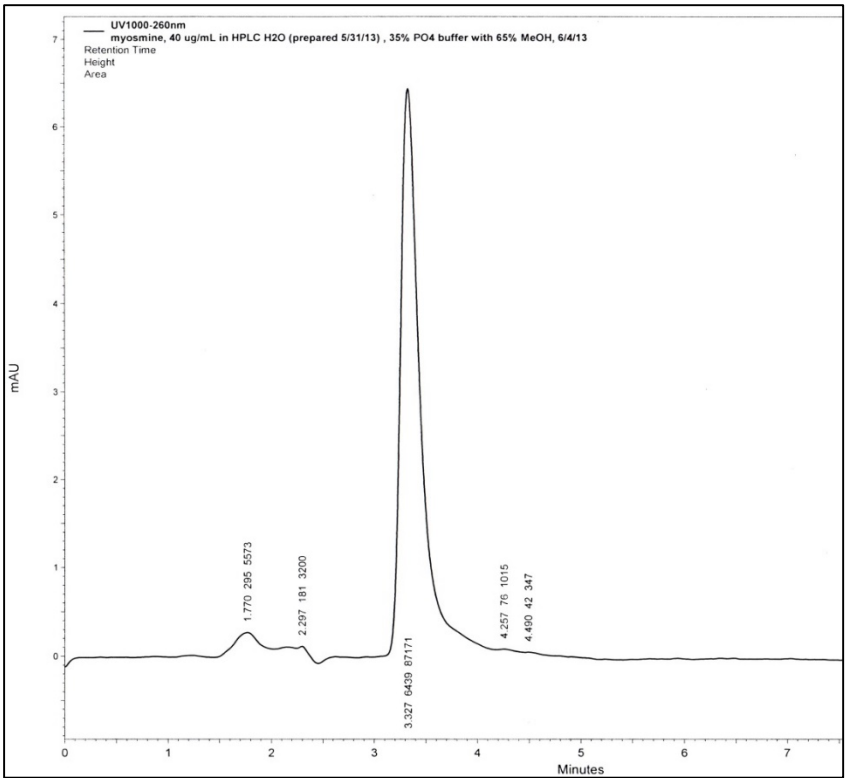

Figure 9: HPLC Chromatogram of Myosmine Using Thermo Finnigan HPLC.

Retention time = 3.327 min.

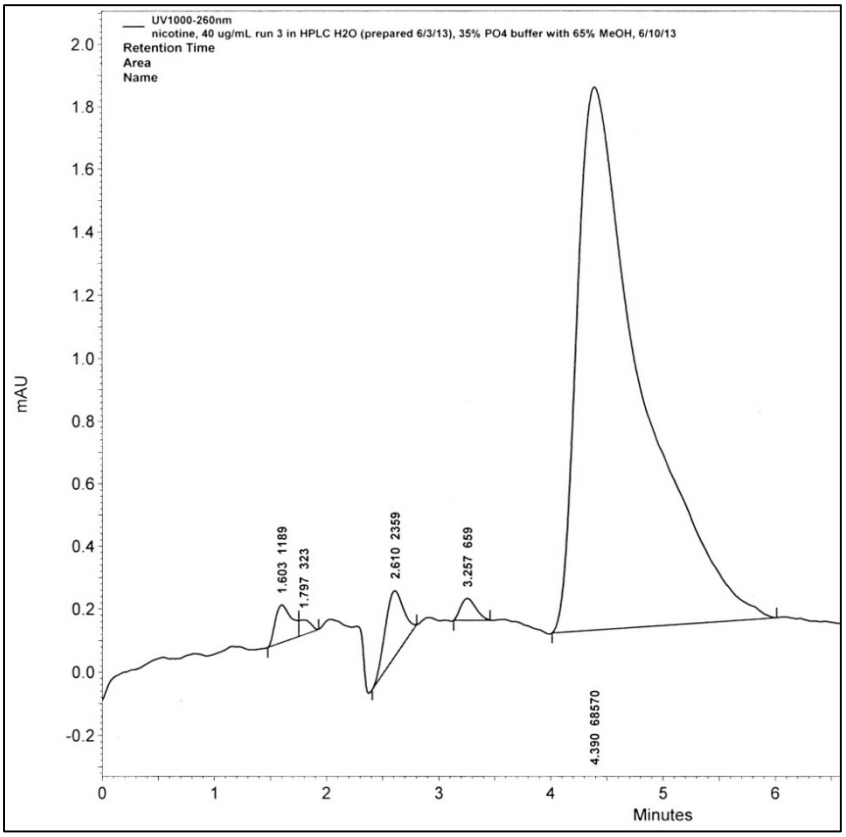

Figure 10: HPLC Chromatogram of Nicotine Using Thermo Finnigan HPLC.

Retention time = 4.390 min.

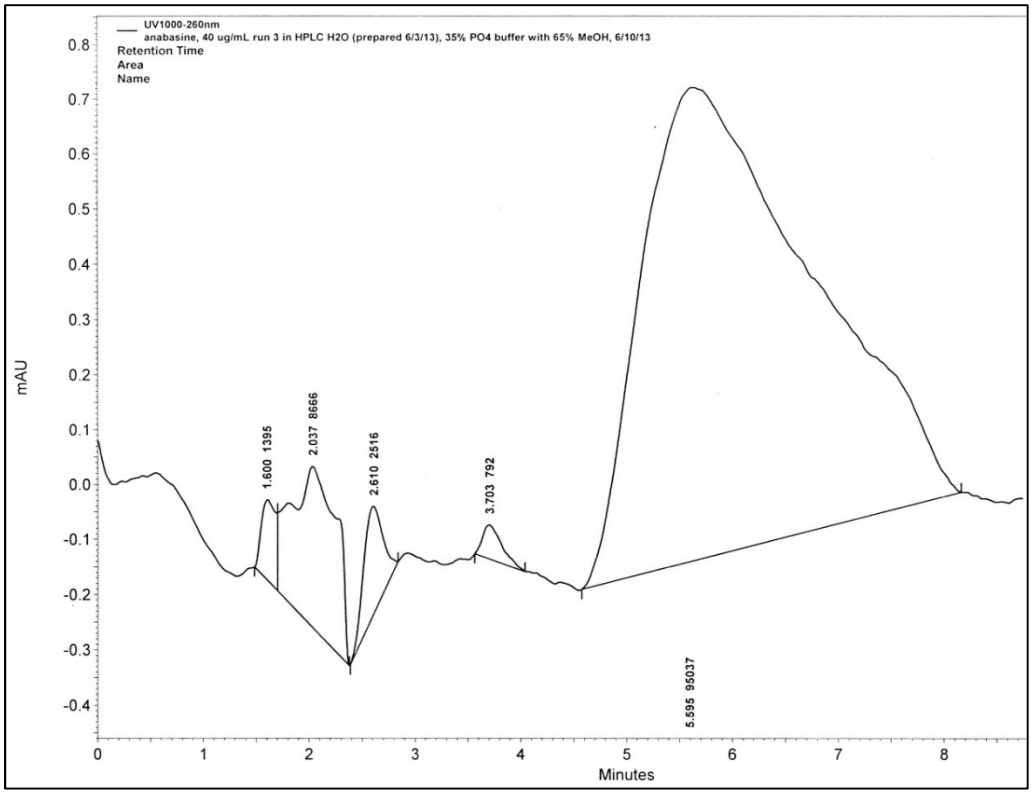

Figure 11: HPLC Chromatogram of Anabasine Using Thermo Finnigan HPLC.

Retention time = 5.595 min.

Figures 10 and 11 effectively demonstrate some of the difficulties encountered during this project while working with the alkaloids nicotine and anabasine: these alkaloids in particular tended to have stronger retention characteristics on the column. This was evident by the broader peaks as the retention time was increased when using the Thermo Finnigan instrument. This is possibly due to the molecular structures and/or pK_a values of these molecules. Both molecules have relatively high pK_a values compared to the alkaloids cotinine and myosmine (see Figure 2). An equilibrium between the ionized and unionized forms of these molecules located at the alicyclic base (that is, the nitrogen located in the non-aromatic ring) may exist at the specified

buffer pH, leading to broadened peaks. In addition, nicotine and anabasine yielded unusually low signals with peak heights of less than 2 mAU—this result indicates a low signal-to-noise ratio for these compounds.

In contrast, cotinine and myosmine demonstrate desirable retention characteristics with this elution scheme. As shown in Figures 8 and 9, the compounds exhibit sharp, well-defined peaks which appear after the void time indicated by the small peaks which appear first on the chromatogram.[68]

Adjustments to the percentage of organic solvent were attempted following the development of this scheme in an effort to improve the retention characteristics of anabasine and nicotine; however, this was often accompanied by little to no retention of the more acidic compounds myosmine and cotinine. Further adjustments to buffer pH, buffer concentration, and organic solvent composition were similarly made as further schemes were developed. The quantitative characteristics of these elution schemes are given in Table 2; qualitative comments regarding these schemes are given in Table 3.

Table 2: Quantitative Data for Attempted Elution Schemes using Thermo Finnigan HPLC									
Mobile Phase No.*	Retention Times (min)				Correlation Coefficient (R^2) Value				λ (nm)
	Cotinine	Myosmine	Nicotine	Anabasine	Cotinine	Myosmine	Nicotine	Anabasine	
1									260
2									260
3	2.632	3.392† 3.305‡	4.384	5.502	0.9787	0.3662† 0.9956‡	0.9971	0.9172	260 & 234
4			3.729	4.167					260
5	2.536	3.042	3.806	3.767					260
6	2.720	3.711	5.563	5.431	0.9999	0.9999			260
7			2.533	2.651				0.9854	260
8	2.54	3.505	8.802	13.631					260
9			2.263	2.233					260
10			2.042	2.07			0.9786	0.9892	260
11			2.072	2.085					260
12			7.218	3.662					260
13			2.955	2.356					260
14	2.843	4.6185	2.986	2.396					260

*The mobile phase number refers to those listed in Table 1.

†These retention times refer to those obtained at a detection wavelength of 260 nm.

‡These retention times refer to those obtained at a detection wavelength of 234 nm.

For mobile phases 1 and 2, the alkaloids could not be resolved from the chromatogram.

Table 3: Qualitative Data for Attempted Elution Schemes using Thermo Finnigan HPLC	
Mobile Phase No.*	Comments
1	No resolution
2	No resolution
3	Well-defined for cotinine and myosmine; broad nicotine and anabasine peaks
4	significant tailing and split peaks for nicotine and anabasine
5	Tailing nicotine peak; broad anabasine peak
6	Well-defined for cotinine and myosmine; poor for nicotine and anabasine
7	Well-defined peaks but poor retention
8	Well-defined for cotinine and myosmine; broad peaks for nicotine and anabasine
9	Well-defined peaks but poor retention
10	Well-defined peaks but poor retention
11	Well-defined peaks but poor retention
12	Broad and poorly defined peaks
13	Well-defined with some tailing; poor retention
14	Split peaks for all analytes

*The mobile phase number refers to those listed in Table 1.

Upon purchasing the Agilent HPLC, gradient schemes could again be developed in order to counter the general elution problem. A combination of the previously attempted elution schemes with the best retention characteristics using the Thermo Finnigan instrument led to those attempted on the Agilent instrument.

The initial scheme used 35% acetonitrile and 65 % 25 mM pH 7.9 phosphate buffer run isocratically. Though peaks were narrow and well-defined for all alkaloids, poor retention of cotinine and myosmine was observed—these compounds were detected almost immediately after injection.

To combat this problem and avoid an overlap with the column's void time, the organic solvent was switched from acetonitrile to methanol. This resulted in greater retention without sacrificing the overall peak shape. Figure 12 demonstrates the retention characteristics of myosmine and cotinine in 35% methanol and 65% 25 mM pH 7.9 phosphate buffer. Calibration curves were constructed for all four alkaloids using this isocratic method; these are given in Figures 13-16.

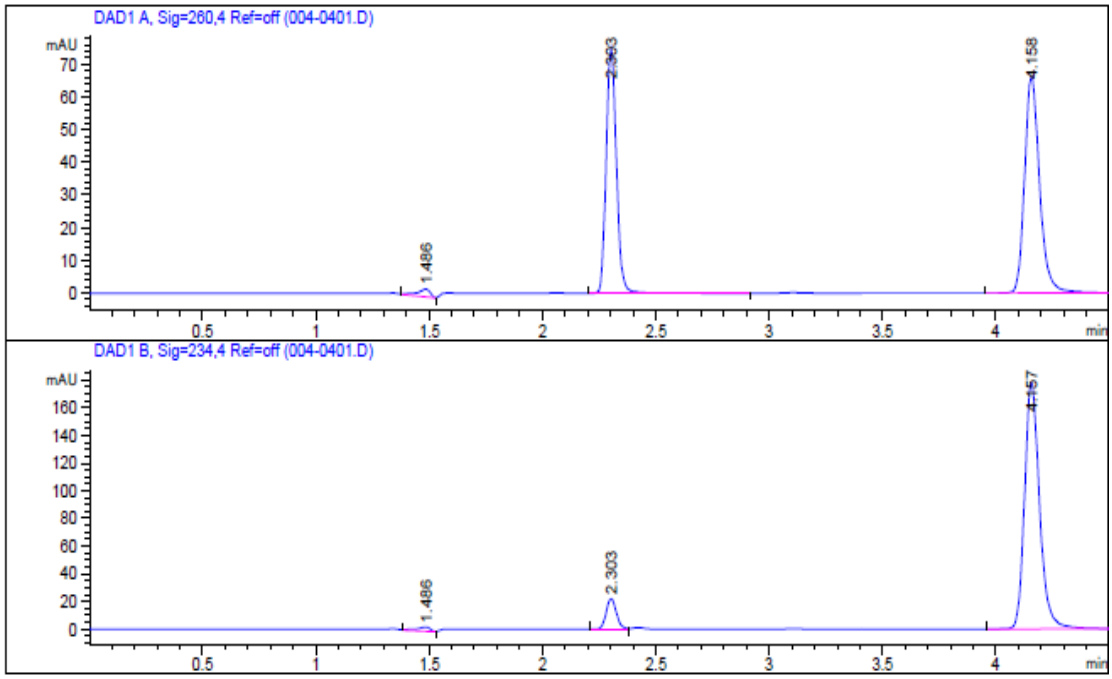

Figure 12: Chromatogram of Cotinine and Myosmine, 65/35 pH 7.9 Phosphate Buffer/MeOH. Cotinine = 2.303 min., Myosmine = 4.157 min.

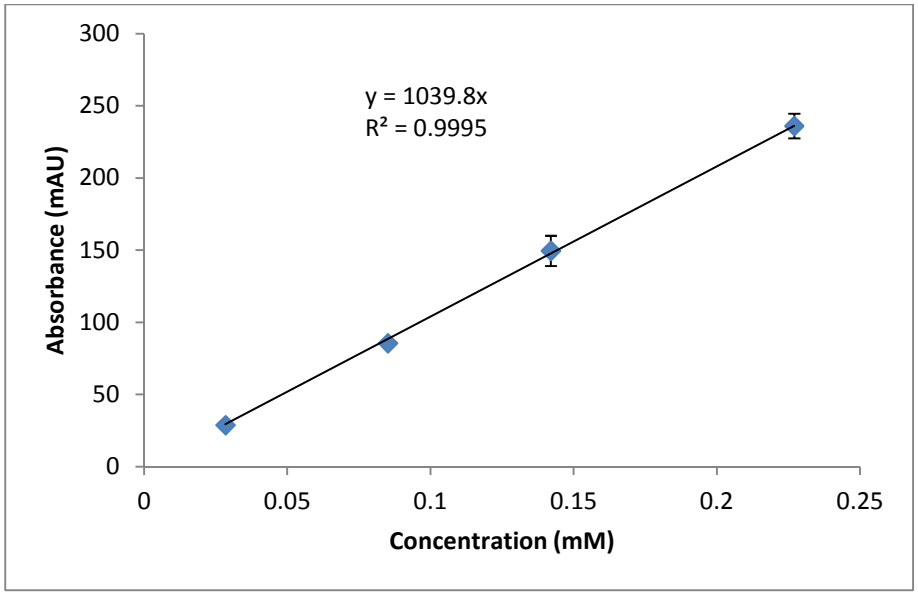

Figure 13: Calibration Curve of Cotinine at 260 nm, 65/35 pH 7.9 Phosphate Buffer/MeOH

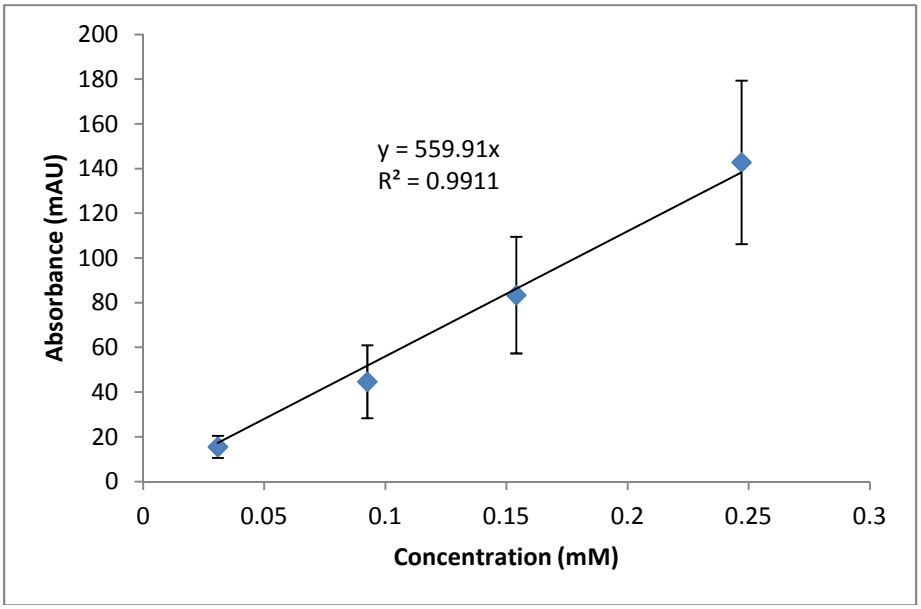

Figure 14: Calibration Curve of Anabasine at 260 nm, 65/35 pH 7.9 Phosphate Buffer/MeOH

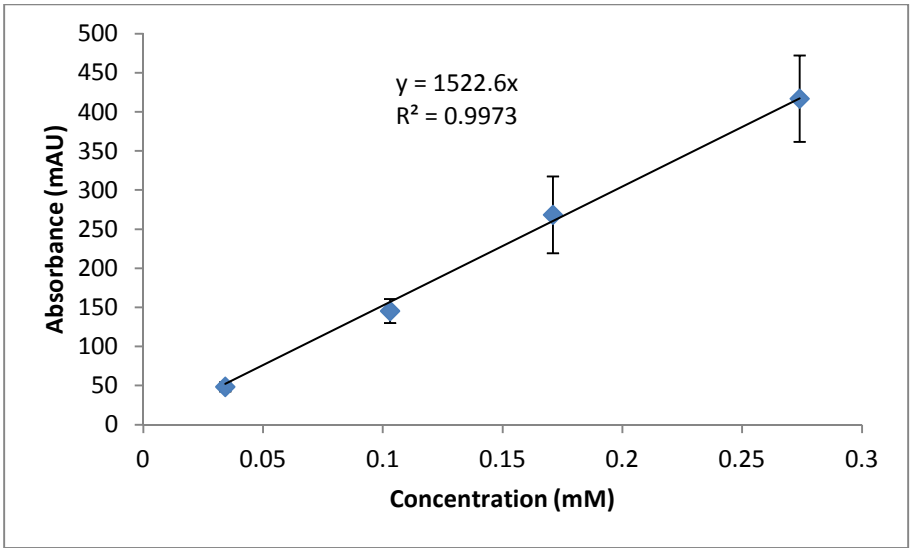

Figure 15: Calibration Curve of Myosmine at 260 nm, 65/35 pH 7.9 Phosphate Buffer/MeOH

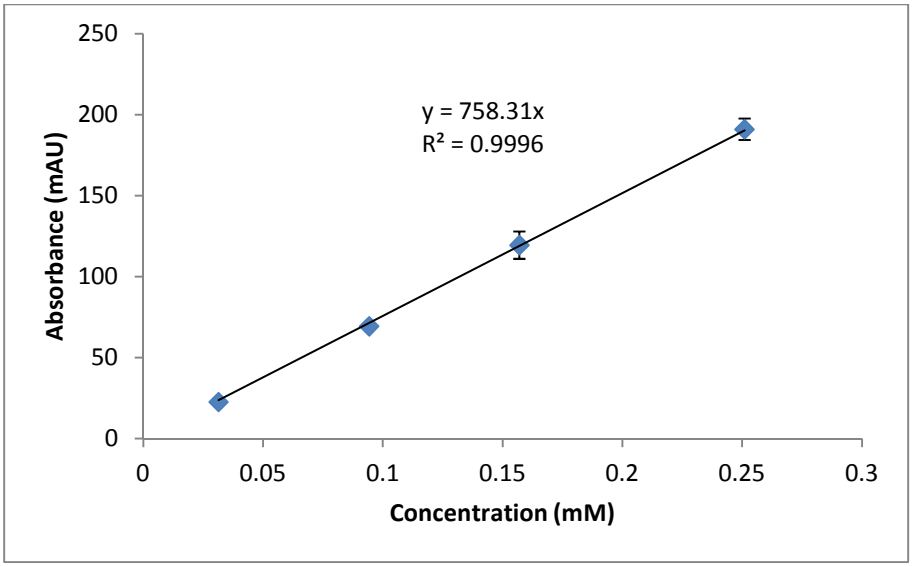

Figure 16: Calibration Curve of Nicotine at 260 nm, 65/35 pH 7.9 Phosphate Buffer/MeOH

Upon finding desirable retention characteristics using this isocratic method, mixed standards containing nicotine, anabasine, cotinine, and myosmine were

injected under same solvent conditions. The results are shown in Figure 17. According to the figure, myosmine and cotinine again demonstrate excellent narrow peaks with this method. Though anabasine and nicotine (eluted at 4.017 minutes and 5.811 minutes, respectively) exhibit broader peaks in comparison, the reproducibility and overall peak shape was greatly improved when compared to results from the Thermo Finnigan instrument. A significant overlap in retention between anabasine and myosmine exists with this method, however—further adjustment was needed to resolve these compounds.

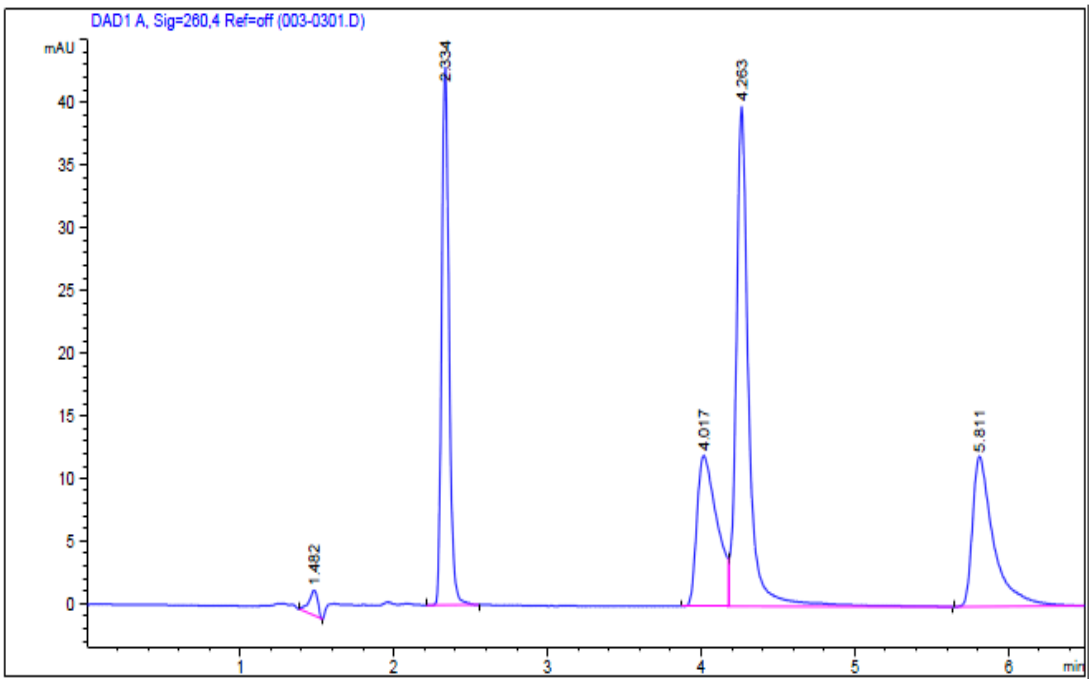

Figure 17: Chromatogram of Alkaloids, 65/35 pH 7.9 Phosphate Buffer/MeOH Cotinine = 2.334 min., Anabasine = 4.017 min., Myosmine = 4.263 min., Nicotine = 5.811 min. Note the co-elution of myosmine and anabasine.

In order to resolve the peaks corresponding to anabasine and myosmine, the percentage of methanol in the mobile phase was increased to 50%. An isocratic method of 50% 25 mM pH 7.9 phosphate buffer and 50% methanol was used to elute a mixed standard containing myosmine and anabasine. The results are shown in Figure 18.

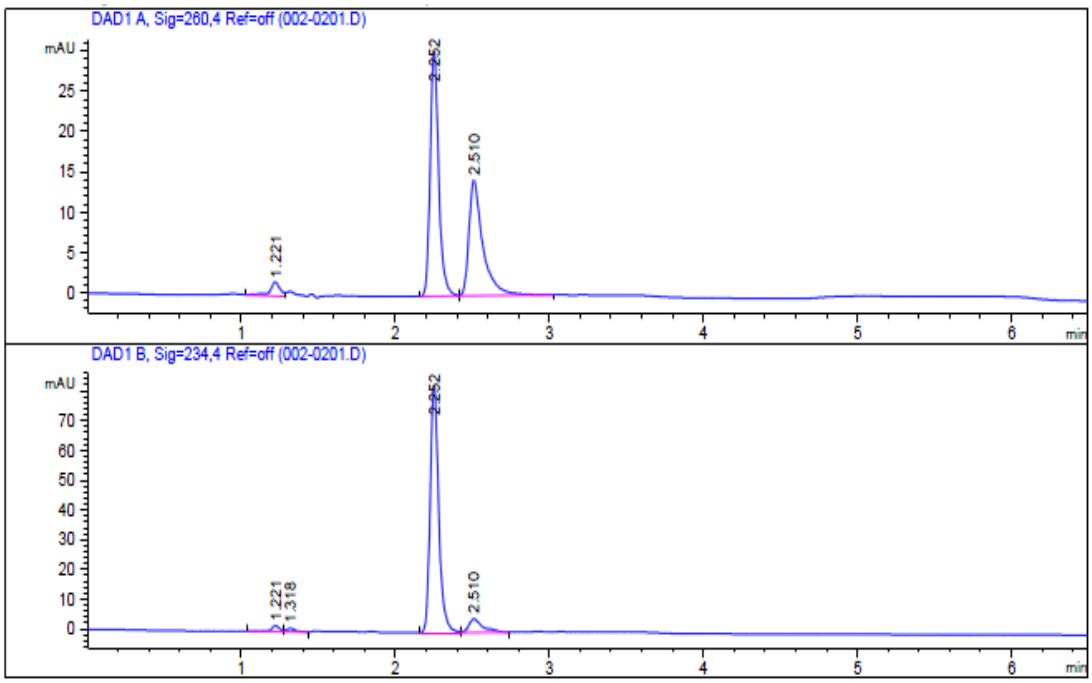

Figure 18: Chromatogram of Anabasine and Myosmine, 50/50 pH 7.9 Phosphate Buffer/MeOH.

Myosmine = 2.252 min., Anabasine = 2.510 min. The top chromatogram used a wavelength of detection of 260 nm; the bottom chromatogram used a wavelength of detection of 234 nm.

In order to gauge the efficacy of this method, the resolution between these peaks was calculated. Chromatographic resolution may be calculated using Equation 4:

$$R_S = \frac{2\Delta Z}{(W_A + W_B)} \qquad \text{Eq. 4}$$

where R_s is resolution, ΔZ is the difference in time between the peaks of interest, W_A is the width of the first eluted peak, and W_B is the width of the second eluted peak.[68] Since the widths W and the time difference Z are both given in units of time, R_s is a unitless quantity. The average resolution measured between these peaks was measured as 3.421 ± 0.022. A resolution greater than or equal to 1.5 is typically deemed adequate for baseline separation between two peaks.[70]

Following these results, a gradient scheme was adapted by combining the two above methods—the concentration of the organic solvent methanol was gradually increased prior to the elution of the compounds myosmine, anabasine, and nicotine. This resulted in a shift in retention order between anabasine and myosmine, causing the more acidic alkaloid myosmine to elute before anabasine. Separation between myosmine and anabasine was achieved, resulting in a simple gradient scheme only 6.5 minutes in length. This scheme is much shorter in comparison to the 30 minute run time required to separate alkaloids in the FDA-funded study discussed previously.[6] The results of this method are demonstrated in Figure 19.

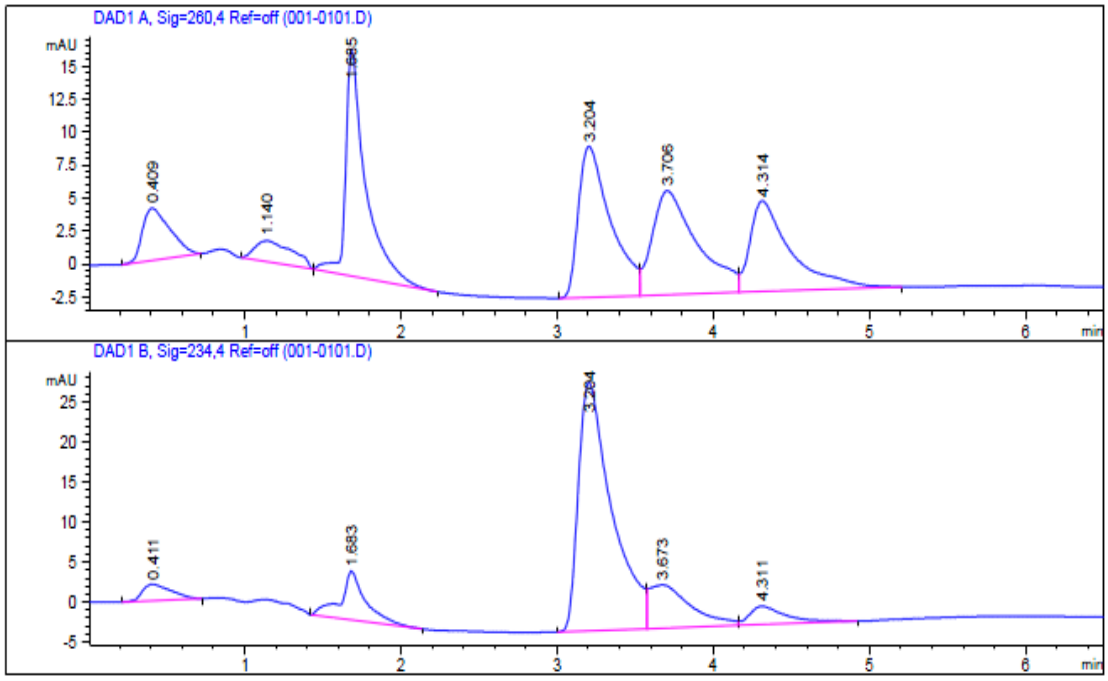

Figure 19: Chromatogram of Alkaloids:

Cotinine = 1.685 min., Myosmine = 3.204 min., Anabasine = 3.706 min., Nicotine = 4.314 min. Alkaloids were analyzed using the following scheme: Gradient of 65/35 pH 7.9 Phosphate Buffer/MeOH to 50/50 pH 7.9 Phosphate Buffer/MeOH from 0 to 3 minutes; Isocratic 50/50 pH 7.9 Phosphate Buffer/MeOH from 3 to 6.5 min.

The overall peak shapes shown in Figure 15 suffered due to the presence of contaminants on the chromatographic column. Repeated washings of the column with HPLC-grade water and methanol led to a gradual improvement from split, broad peaks to those shown above in Figure 19. Research was suspended in October of 2013 following maintenance on the chromatograph injection port and a need to clear obstructions from the eluent lines and column.

FUTURE WORK

The developed elution scheme may be improved by reducing the effects seen from ionization of analytes due to a shift in pH. The addition of the mobile phase buffer to the alkaloid samples prior to injection may enhance separation as well as allow the alkaloids to equilibrate to the buffered portion of the mobile phase before analysis. Existing literature demonstrates that low concentrations (approximately 0.1%) of strong acids such as trifluoroacetic acid may be added to the alkaloids prior to injection to control the pH.[83] This has limited efficacy for more basic analytes; however, it has been used to control the mobile phase pH and selectivity for some slightly ionizable compounds such as proteins. Care must be taken when pursuing this option so as not to lower the mobile phase pH below the acceptable range for the column (for the Agilent Poroshell column, this range is 2.0 – 9.0).[80, 84]

The original goal of this study was to test a variety of e-cigarette filling solutions using a predetermined HPLC elution scheme found in the literature. During the course of this work, instrument issues such as those discussed previously led to a shift in focus from a quality control study regarding the alkaloid content in e-cigarettes to a study in method development for e-cigarette alkaloids using mixed standards. In the future, e-cigarette filling solutions will be diluted to 1/1000 of their original concentrations and run using the HPLC elution scheme described in Figure 19. The concentrations of each alkaloid in the refill solutions will be reported along with their statistical significance. Attention will be given to the concentration of

nicotine found experimentally as it compares to the concentration advertised by the manufacturer and amounts reported by pre-existing research.

CONCLUSION

The goal of this work was to add to the growing amount of knowledge regarding e-cigarette quality, safety, and efficacy by developing an HPLC elution scheme capable of separating and quantifying nicotine and the related e-cigarette alkaloids anabasine, cotinine, and myosmine. A simple gradient elution scheme was developed with the following specifications: a transition from 65% to 50% 25 mM pH 7.9 phosphate buffer was accompanied by an increase from 35% to 50% methanol from 0 to 3 minutes, followed by isocratic elution from 3 to 6.5 minutes with 50% 25 mM pH 7.9 phosphate buffer and 50% methanol. This elution scheme was sufficient to resolve all alkaloids with reasonable peak shape and separation. The method will be used to quantify the amount of nicotine and related alkaloids in e-cigarette filling solutions purchased from online vendors. The experimental nicotine concentrations may then be compared to the manufacturers' reported nicotine concentrations to provide an assessment of the quality control surrounding e-cigarette filling solutions.

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