Developing a Method to Extract Alkaloids from the Secretions of Salamanders Found in Northwestern North Carolina

By

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I. Abstract

Many species of salamanders secrete noxious chemicals as a defense mechanism. Two salamanders found in western North Carolina, *P. yonahlossee* and *P. montanous*, belong to the same genus, but show vastly different coloring. *P. yonahlossee* displays a bright red dorsal patch, while *P. montanous* is uniformly grey. Recent research conducted in the Biology Department at Appalachian State University using clay models to represent salamanders showed that predatory birds avoided models with bright red spots, suggesting that the defense mechanism was effective. It is not known, however, if the *P. yonahlossee* secrets noxious substances, or if the red patch is simply a form of mimicry. The goal of this work is to determine if the *P. yonahlossee* secrets noxious substances. Recent research conducted on poisonous frogs suggests that the noxious compounds in salamanders could be alkaloids. I used nicotine as a model alkaloid and compared the ability of liquid-liquid extraction (LLE) and solid phase extraction (SPE) to recover alkaloids from salamander secretion. GC/MS was used to quantify all extracts. Although previous students optimized the LLE method, we have been unable to reproduce their results and have done experiments to determine if nicotine is lost in the evaporation step of the extraction. We used two different GCs and determined that one of them provides cleaner chromatograms, probably because it has fewer users. Although our samples have aged in the freezer and our proposed method is only semi-quantitative, this research has the potential to answer important questions about salamanders in western North Carolina that can be applied to other regions. In addition, to our knowledge, no other methods in the literature have used SPE to recover alkaloids from salamander secretion, so there is potential to contribute a new, robust method.

II. Introduction

Defensive Behaviors of Salamanders

Many amphibians ward off predators using defense mechanisms such as defensive postures, secretion of noxious chemicals, or tail autonomy, where the amphibian self-amputates its tail. Salamanders exhibit many of these defense mechanisms, which appear to be species dependent. The defensive behavior and skin secretions of the salamander, *Ambystoma gracile*, were investigated by Brodie (1969). When attacked, *A. gracile* head-butted and tail lashed at the predator, in order to bring its secretion glands in contact with the predator. Many of *A. gracile's* predators were repulsed by the smell and taste of the secretions. When a predator came in contact with the secretion, the water insoluble secretion hardened over the predator's eyes and caused inflammation with labored breathing. Rats that were injected with the secretions experienced sensitivity to light and touch, loss of righting, instability, and convulsions before death. The author even placed a drop of the secretion on his tongue and experienced drying, loss of sense, and burning which lasted for 15 minutes. These observations suggested that other salamanders may possess similar defense mechanisms, and their secretions may have similar effects.

The European fire salamander, or *Salamandra salamandra terrestris*, also secretes noxious chemicals as a defense mechanism. The alkaloids, samandarine and samandarone (Figure 1), were identified in the skin secretions, liver, testes, ovaries, and skin tissue of the fire salamander using a simple chloroform extraction and analyzed by gas chromotography/mass spectrometry. The presence of alkaloids in the internal organs, as opposed to the stomach lining and intestines, suggested that the alkaloids were synthesized internally and not obtained from the diet (Mebs).

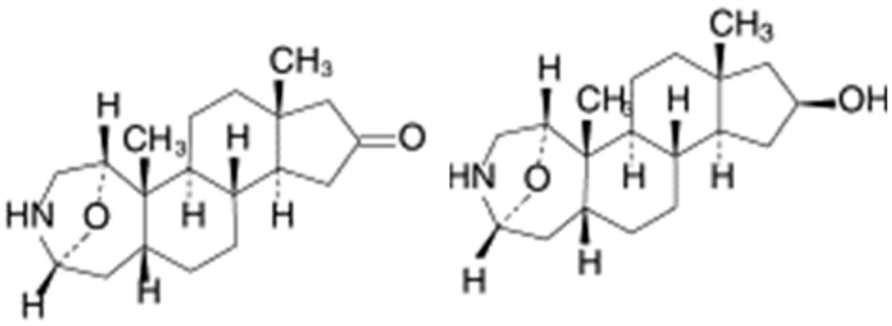


Figure 1: Chemical Structures of the Alkaloids, Samandrone (left) and Samandarine (right), found in the *S. salamandra terrestris*

Some salamanders, such as *Batrachoseps attenuatus*, use their secretions as "glue" to immobilize predators. For example, when attacked by a snake, *B. attenuatus'* secretions harden over top of the snake—immobilizing it—allowing the salamander to wiggle free. (von Byron, Toxicon, 2017). Several salamanders, including *Plethodon glutinosus, Plethodon shermani, Ambystoma opacum,* and *Ambystoma maculatum* have secretions that work similarly to those of *B. attenuatus*. The secretions of these salamanders were recently evaluated for use as natural bioadhesives and compared to the toxic secretions from *Pleurodeles waltl*, *Triturus carnifex, Pseudotriton ruber, Tylototriton verrucosus,* and *Salamandra salamandra*. The secretions of *A. opacum* and *P. shermani* were not only biocompatible in cell cultures, they also stimulated cell growth, most likely because the secretions were rich in nutrients such as proteins, carbohydrates, and lipids. The secretion of *T. verrucosus*, which is known to be toxic, also stimulated growth in cell cultures, possibly because a wound-healing peptide found in the salamander's skin, tylotion, was also present in the secretion. If the toxicity of *T. verricosus*' secretion could somehow be inactivated, it would have great potential for wound healing (von Byern).

<u>Aposematism and Salamanders in Western North Carolina</u>

Aposematism is a common defense mechanism in which bright or contrasting colors and patterns are used to warn predators of toxicity. Predators either innately know to avoid these bright

colors, or over time learn to associate brightly colored prey as poisonous. For example, wasps display bright contrasting black and yellow colors to warn off predators. The bright blue wings of the pipevine swallowtail are also a warning for birds, wasps, frogs, and other predators.

The salamander, *Notophthalmus viridescens,* shown in Figure 2, lives in western North Carolina and other regions in eastern North America. Brightly colored with red-orange skin, *N. viridescens* secretes the neurotoxin, tetrodotoxin, shown in Figure 3. A similarly colored salamander found in western North Carolina, *Plethodon yonahlossee,* displays a bright red dorsal patch (Figure 2) and secretes when under attack, but the compounds in the secretion have not been identified. It is possible that the secretions of *P. yonahlossee* are harmless and that the red dorsal patch evolved to mimic the color of *N. viridescens,* but the secretions could also be toxic. Another salamander native to western North Carolina, *Plethodon montanus*, (Figure 2) is entirely grey colored, but belongs to the same genus as *P. yonahlossee*. The effect of salamander color on the amount of predatory attacks by birds was recently studied (Winebarger). Clay models representing *P. montanus* (gray) and *P. yonahlossee* (red dorsal patch) were created and hundreds of them were placed in an open field. The number of attacks on each model were counted over a given time period. Predators were more likely to attack the gray model (representing *P. montanus*), which suggested that the red patch is an effective defense mechanism in *P. yonhalossee.*

Figure 2: ***N. viridescens*** **(top)*, P. yonahlossee*** **(middle) and** ***P. montanus*** **(bottom)**

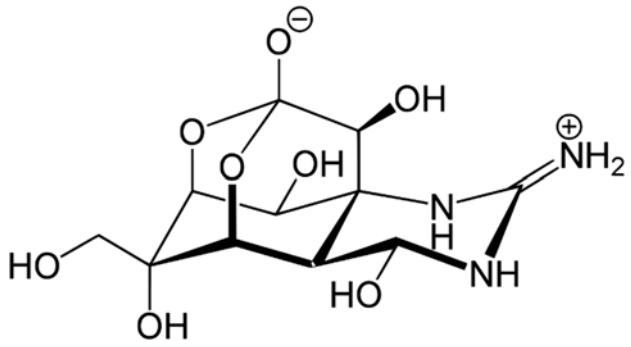


Figure 3: Chemical Structure of Tetrodotoxin, found in the toxic salamander *N. viridescens*

Secretions from Frogs

Although little is known about secretions from salamanders—especially those in western North Carolina—much is known about the secretions from poison frogs. In 1980, roughly 100 different alkaloids belonging to the family, batrachotoxin, were discovered in the poison frog, *Dendrobates tricolor* (Figure 4). It is thought that batrachotoxins affect the nerves and muscles of predators. Since then, more than 850 different lipophilic alkaloids have been identified in poison frogs. 64% have been classified as 5,8-disubstuted indolizidines, pumiliotoxins, 5,6,8-trisubstituted indolizidines, tricyclics, and 2,5-disubsituted decahydroquinolines (Figure 4). Approximately 30% of alkaloids in poison frogs remain unclassified. The alkaloids marked with an asterisk in Figure 4 have been detected only in amphibians, and nowhere else in nature. These alkaloids could potentially be present in salamander secretions (Saparito).

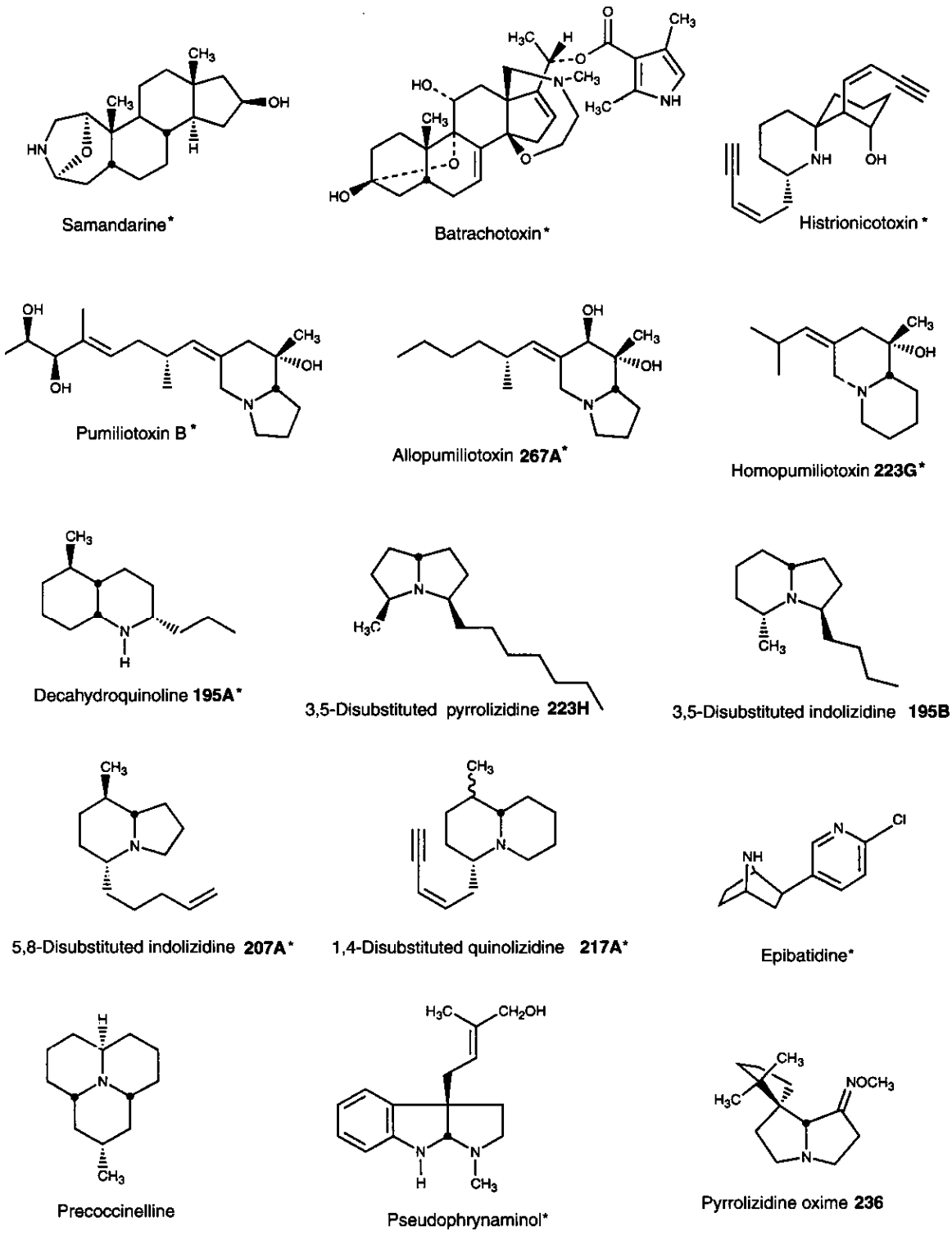


Figure 4: Chemical Structures of the Alkaloids detected in Amphibians

The retention times and mass to charge ratios of alkaloids in frogs have been published. Fifteen alkaloids are commonly found in amphibians: samandarine, batrachotoxin, histionicotoxin, pumilitoxin B, aliopumilotoxin, homopumilotoxin, decahydroquinoline, disubstituted pyrrolizidine, 3,5-disubstituted indolizidine, 5,8-disubstituted indolizidine, 1,4-disubstituted quinolizidine, epibatidine, precoccinelline, pseudophrynaminol, and pyrrolizidine oxime. The names, formulas, molecular weights, and GC/MS retention times of these alkaloids are shown in Table 1. The retention times can be used identify alkaloids in secretions from salamanders such as *P. yohahlosse*, and the molecular weights can be used to predict mass to charge ratios that could appear in the mass spectra (Saparito).

Table 1: Most Common Alkaloids in Amphibians Retention times were determined using a 30-m fused silica capillary column programmed from 100°–280° at 10°C/min. The values have been normalized, based on the values of well-known alkaloids also present in the extracts.

Alkaloid Name	Chemical Formula	Molecular Weight (g/mol)	Retention Time (minutes)
Samandarine	$C_{19}H_{31}NO_2$	305	No information
batrachotoxin	$C_{31}H_{42}N_2O_6$	538	Group of alkaloids
Histionicotoxin	Group of alkaloids, information varies		
pumilitoxin B	$C_{19}H_{33}NO_3$	323	17.38
aliopumilotoxin	$C_{16}H_{29}NO_2$	267	12.68
homopumilotoxin	$C_{14}H_{25}NO$	223	8.83
decahydroquinoline	$C_{13}H_{25}N$	195	7.20- cis isomer 7.38- trans isomer
disubstituted pyrrolizidine	$C_{15}H_{29}N$	223	8.91
3,5-Disubstituted indolizidine	$C_{13}H_{25}N$	195	6.61, 6.68
5,8-Disubstituted indolizidine	$C_{14}H_{25}N$	207	7.90
1,4-Disubstituted quinolizidine	$C_{15}H_{23}N$	217	9.48
epibatidine	$C_{11}H_{13}ClN_2$	208	12.35
precoccinelline	$C_{13}H_{23}N$	193	6.91
pseudophrynaminol			
pyrrolizidine oxime	$C_{14}H_{24}N_2O$	236	10.50

Extraction of Alkaloids from Amphibian Skin

The most common technique used to extract alkaloids from amphibians is solvent extraction. Saparito used liquid-liquid extraction (LLE) to extract alkaloids from the skin of poison frogs that were collected during wet and dry seasons in Panama. Samples collected in the dry season were macerated, dissolved in methanol, diluted in water, and then extracted with chloroform. The layers were concentrated using a rotary evaporator, then acidified in order to protonate the alkaloids before extraction with hexanes. After discarding the hexane layer, the aqueous layer was made basic in order to deprotonate the alkaloids and extracted once more with chloroform. The chloroform layer was then dried with Na_2SO_4, evaporated to dryness, and dissolved in methanol. Samples collected during the wet season were extracted similarly, except that the original extraction with chloroform was omitted. An overview of this procedure is shown in Figure 5.

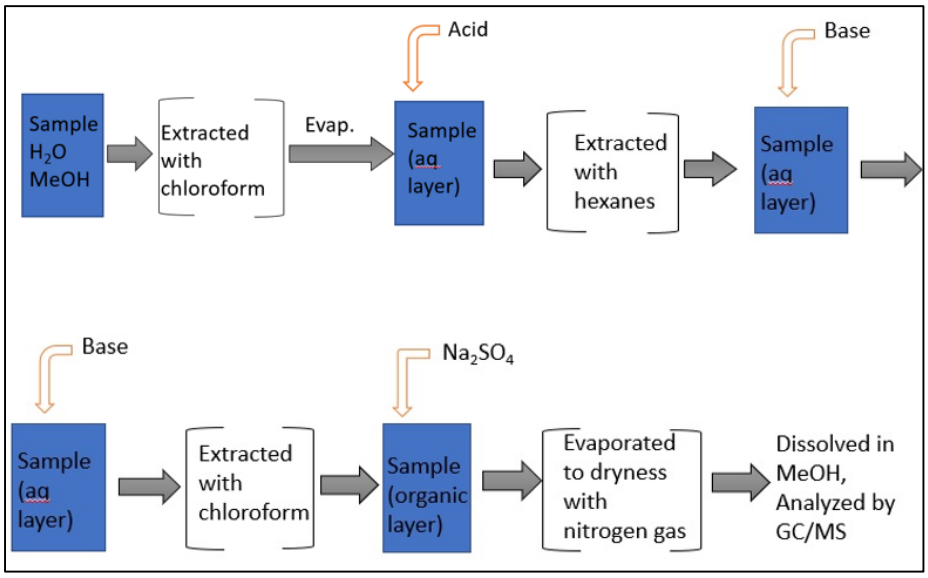


Figure 5: Overview of LLE Method of Extracting Alkaloids from Frog Skins, Saparito (2006), dry season

Saparito's method was modified slightly by Jeckel et al., who used it to analyze the skins of poison frogs collected in Brazil. As shown in Figure 6, nicotine was used as a surrogate. Hydrochloric acid was added to the macerated skin samples to protonate the alkaloids and the solution was extracted

with hexanes. The aqueous layer was treated with $NaHCO_3$ to deprotonate the alkaloids, then extracted with ethyl acetate, dried with Na_2SO_4, evaporated to dryness, and dissolved in methanol. This method differed from Saparito in small ways, such as a nitrogen evaporator being used instead of a rotary evaporator to evaporate the solution, and in the use of ethyl acetate instead of chloroform.

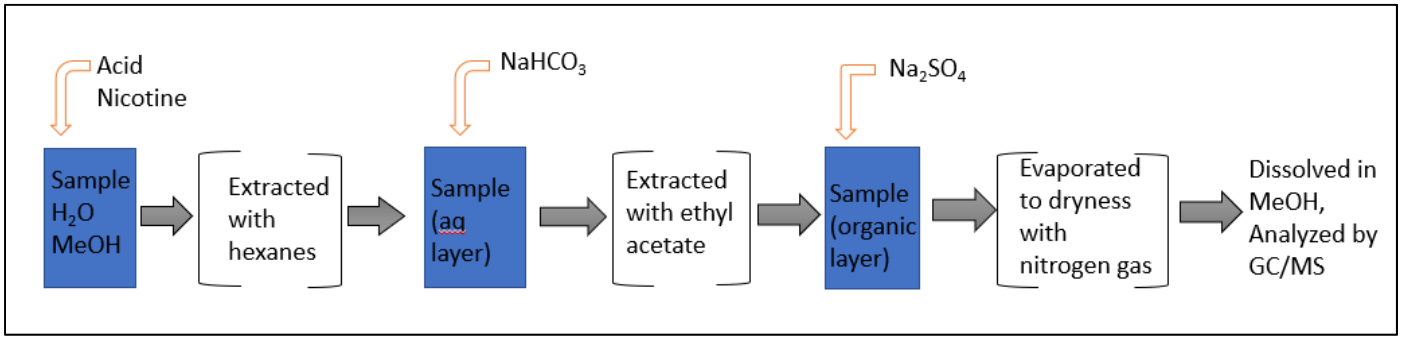


Figure 6: **Overview of LLE Method of Extracting Alkaloids from Frog Skins, Jeckel (2015)**

Alkaloids have been extracted from biological samples such as blood and urine using solid phase extraction (SPE). Here, samples were mixed with hydrochloric acid and acetonitrile, vortexed, sonicated, and centrifuged. The alkaloids were then extracted using a strong cation exchange column that was conditioned with methanol and water. Samples were loaded on the columns, washed with a methanol water solution, and eluted with an ammonia-methanol solution. The extracts were then analyzed using HPLC-MS/MS. SPE could potentially be more efficient at extracting alkaloids from salamander secretions without destroying or losing any analyte, but would probably be more expensive. To our knowledge, no one has used SPE to extract alkaloids from amphibian secretions.

<u>Developing a Method to Extract Alkaloids from Secretions of Salamanders in Western North Carolina</u>

Most of the previous work on this project was performed by former undergraduate chemistry major, Alexander Wilson. Wilson developed a method for alkaloid extraction that was based on the methods used by Saparito and Jeckel. Solvent volumes were scaled down to reduce waste and increase

efficiency. Various surrogates—including nicotine, quinine, and quinoline—were explored, but nicotine was most suitable. Acid and base concentrations were optimized to protonate and deprotonate nicotine. Recognizing that the salamander secretion was not pure—it also contained water and methanol as a result of the sample collection process—Wilson attempted to quantify the water present in the secretion using GC/MS, which ultimately proved to be too difficult. The current sample collection method, therefore, limits our results to being semi-quantitative, because we do not know how much secretion, water, and methanol are in the actual sample. A future goal for the project, therefore, must be to optimize the sample collection method.

III. Purpose

Although our method is only semi-quantitative, and our secretions have been stored in the fridge for three years, this work could lead to a robust method for alkaloid extraction from biological secretions. The compounds in salamander secretions from western North Carolina could be identified. Broadly speaking, this work could discover previously unknown alkaloids with potential medicinal or biological applications. The purpose of this work is to compare the alkaloids found in *P. yonahlossee* and *P. montanus* using LLE and GC/MS. Based on the previous studies of amphibians and salamanders, I hypothesize that secretions from *P. yonahlossee* (with its red dorsal patch) will produce toxic effects, and therefore contain alkaloids, similar to those reported in the literature, but that *P. montanus* (uniformly gray) will not contain alkaloids or produce toxic effects. The liquid-liquid extraction and GC/MS methods developed by Wilson were used initially. A new sample collection method for the collection of fresh samples has also been recommended.

IV. Experimental

Secretion Sample Collection

P. yonahlossee and *P. montanus* were collected by previous students from university-owned land during the 2015 academic year. The "hassle-bag" technique was used to collect secretions. Briefly, salamanders were placed in plastic bags, wet with water, and hassled (poked) until they released secretions. The secretions were scraped and rinsed off the bag using methanol into 1-mL plastic vials.

Liquid-Liquid Extraction

The extraction methods developed by Jeckel and Saparito were modified as follows. Solvent volumes were scaled down, because our samples were smaller than those used in the literature. Glass vials and pipets replaced round bottom flasks and separatory funnels, and nitrogen gas was used to evaporate solvents instead of rotary evaporation. Scaling down minimized waste and reagents needed to complete the extraction.

In a 7-mL glass vial, 100 µL of 1.0 mg/mL nicotine, 800 µL of methanol, 1 mL of 8 M HCl, and the secretion sample were combined. The vial was vortexed, then extracted five times with 2.5 mL hexanes. The hexane layer was set aside. The aqueous layer was treated with 3 mL of 9 M NH_3, vortexed, and then extracted three times with 3 mL chloroform. The chloroform layer was dried with anhydrous Na_2SO_4. Both the dried chloroform layer and the hexane layer were separately evaporated to dryness using an Organomation Associates N-evap evaporator, then dissolved in 1 mL of methanol and stored in GC vials. Any alkaloids, along with the surrogate, should be found in the GC vial containing the chloroform layer. The concentrations of acid (8 M HCl) and base (9 M NH_3) used here were higher than those used in previous work but were necessary since volumes were scaled down. Again, the concentrations of HCl and NH_3 were optimized in previous work by Wilson.

Gas Chromatography-Mass Spectrometry Procedure

An Agilent Technologies GC/MS equipped with an electron ionization (EI) source and a 5973 Inert mass selective detector, 6890 Network GC system, 30-m VF-5ms column (30m × 0.25 mm × 0.25 µm), and 7683B series injector was used initially. This GC/MS is referred to as the Agilent system from here on. The oven temperature increased from 75-175°C at 10°C/minute. The inlet temperature was 250°C and the helium carrier gas flow rate was 1.0 mL/min. The mass scan range was 50-400 m/z.

A Trace GC Ultra gas chromatograph equipped with an EI source and a triple quadrupole TSQ Quantum XLS mass detector was also used and is referred to from here on as the Thermo system. Chromatographic separation was achieved using a capillary column (30m × 0.32 mm × 0.25 µm, cross-linked 5% diphenyl and 95% dimethyl polysiloxane) from Agilent® and high-purity helium C-60 was used as carrier gas (1.5 mL/min). The initial oven temperature was held for 2 min at 70ºC, increased to 240ºC at 20ºC/min, held for 5 min at 240 ºC , then increased to 310ºC at 50ºC/min, and held for 2 min, giving a total run time of 18.90 min. The injector temperature was set at 270ºC. The temperature of the transfer line was 280ºC. Semi-quantitative analysis was performed in selected ion monitoring (SIM) mode with splitless injection (1 µL). The designated ions were *m/z* 83, 133 and 161 for nicotine. Qualitative analyses were performed in the full-scan mode in the range of *m/z* 50–650.

Preliminary Solid Phase Extraction

Preliminary experiments using solid phase extraction (SPE) were performed on blanks spiked with the surrogate (nicotine). The blanks were acidified using HCl. Blanks contained various volumes of water and methanol to represent the secretion samples. Strata-XL-C 100 µm Polymeric Strong Cation tubes (30mg, 1mL) were used. The SPE tube was conditioned with 1 mL methanol and equilibrated with 1 mL of 50:50 H_2O:MeOH. The sample was loaded and sometimes washed with water and methanol.

The surrogate was eluted by adding 500 µL of NH_4OH in methanol; various concentrations of NH_4OH were investigated. The sample was collected in a collection tube and transferred to a GC vial.

A second SPE method was explored, using the same Strata-XL-C tubes. Here, the tubes were conditioned with 0.5 mL of methanol, then equilibrated with 0.5 mL of 100 mM dipotassium phosphate buffer. The sample was loaded, washed once with 0.5 mL of the buffer and then with 0.5 mL of methanol, and dried for 5 minutes under full vacuum. Nicotine was eluted with 0.5 mL of 5:95 ammonium hydroxide:methanol, and diluted to 1 mL with methanol. These methods are outlined in Table 2.

Table 2 SPE Methods

	Method A	Method B
Condition	1 mL of Methanol	0.5 mL of Methanol
Equilibrated	1 mL of 50:50 MeOH:H_2O	0.5 mL K_2PO_4 buffer
Load	Sample	Sample
Wash	1 mL of Methanol 1 mL of Water	0.5 mL K_2PO_4 Buffer 0.5 mL Methanol
Elute	500 µL NH_4OH in Methanol	0.5 mL of 5:95 NH_4OH:MeOH

V. Results and Discussion

Analysis of Nicotine Standards using the Agilent and Thermo Systems

Prior to any extractions, a 100 mg/L nicotine standard dissolved in methanol was run to determine the retention time and peak area expected in an actual extraction. The standard chromatogram obtained using the Agilent system contained a large peak at 7.5 minutes (Figure 7), and the mass spectrum of this peak (Figure 8) confirmed that it was nicotine. The rising baseline in Figure 7

indicated column bleed, and many impurity peaks can be seen at longer retention times. The MS of one of these contamination peaks is shown in Figure 9. The mass spectrum was examined at the characteristic m/z values for septa and column contamination (42, 73, 149, 207, and 281 as shown in Figure 9), and these peaks were confirmed to be contamination. Column contamination was a recurring problem on the Agilent system, which was used heavily by teaching labs. Eventually, we analyzed several nicotine standards using the Thermo system and obtained much cleaner chromatograms, as shown in Figure 10. The retention time on the Thermo system was a bit shorter, at 6.7 minutes, and linear behavior (R^2=0.99) was observed from 10–100 mg/L (Figure 10).

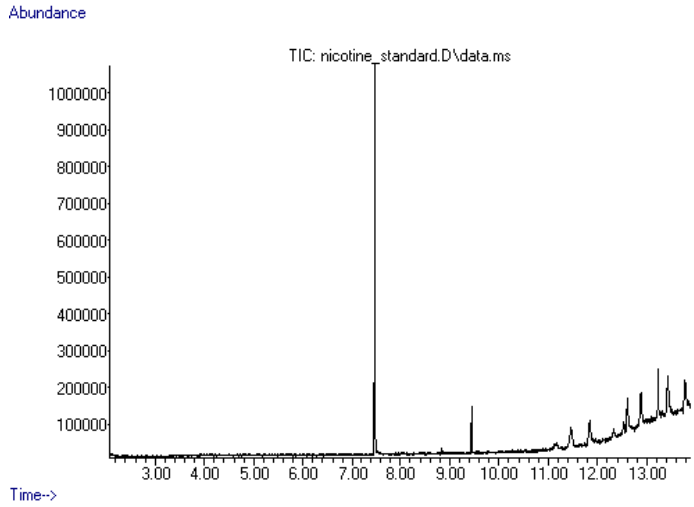


Figure 7 Nicotine Standard, 100 ppm Run on the Agilent system with a 30-m VF-5ms column. The retention time of nicotine was 7.5 minutes.

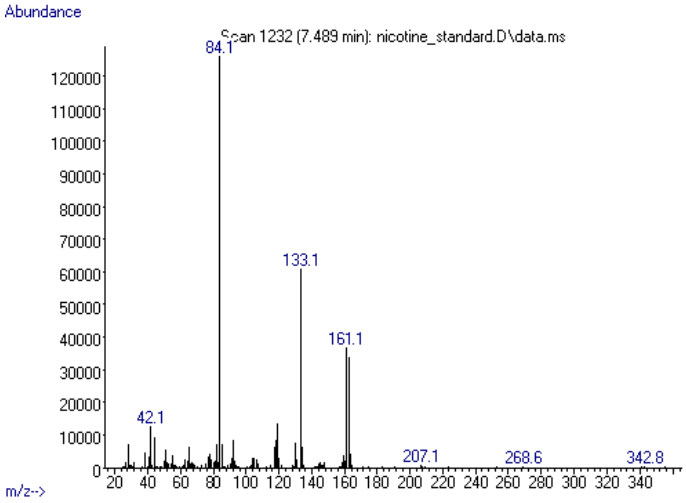


Figure 8 Mass spectrum of 100 ppm nicotine standard Run on the Agilent system with a 30-m VF-5ms column.

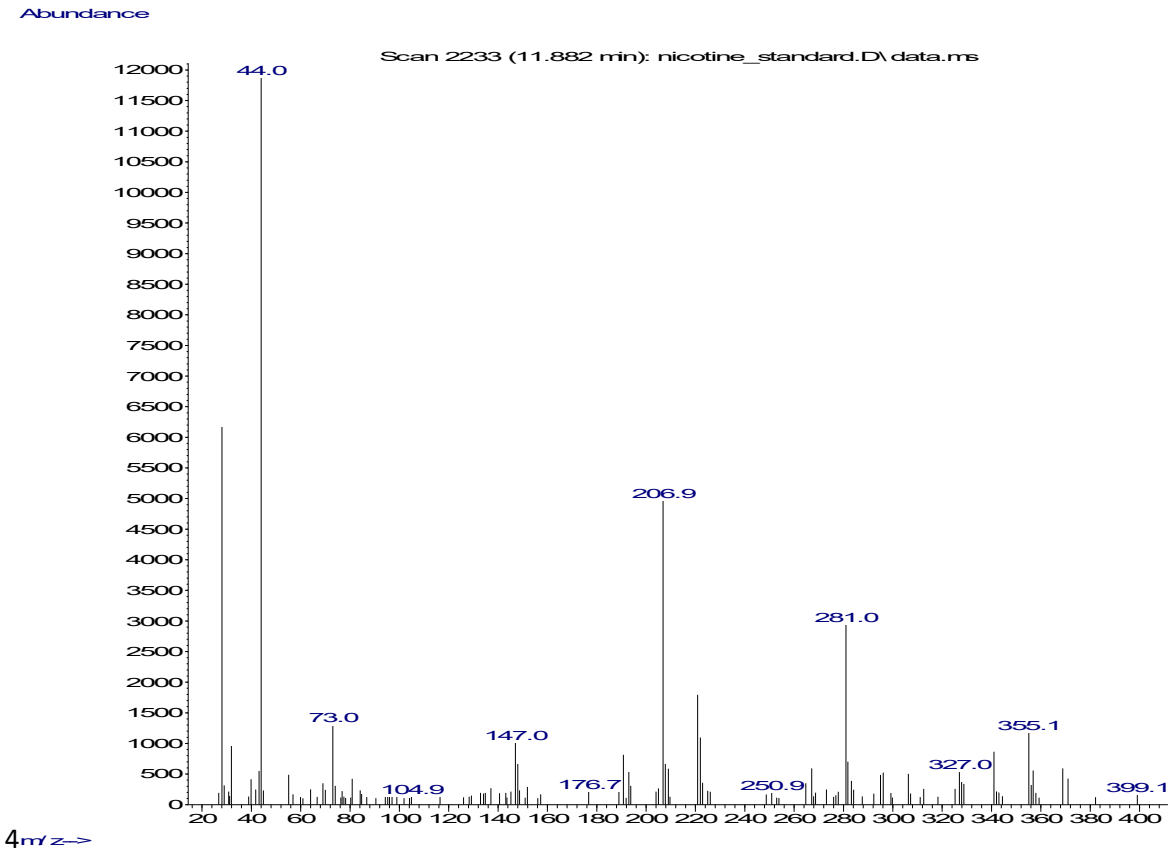


Figure 9 Mass spectrum of column contamination in 100 ppm nicotine standard, peak at 11.7 min Run on the Agilent system with a 30-m VF-5ms column. Contains common contamination ions of 281, 207, 73, and 44 m/z.

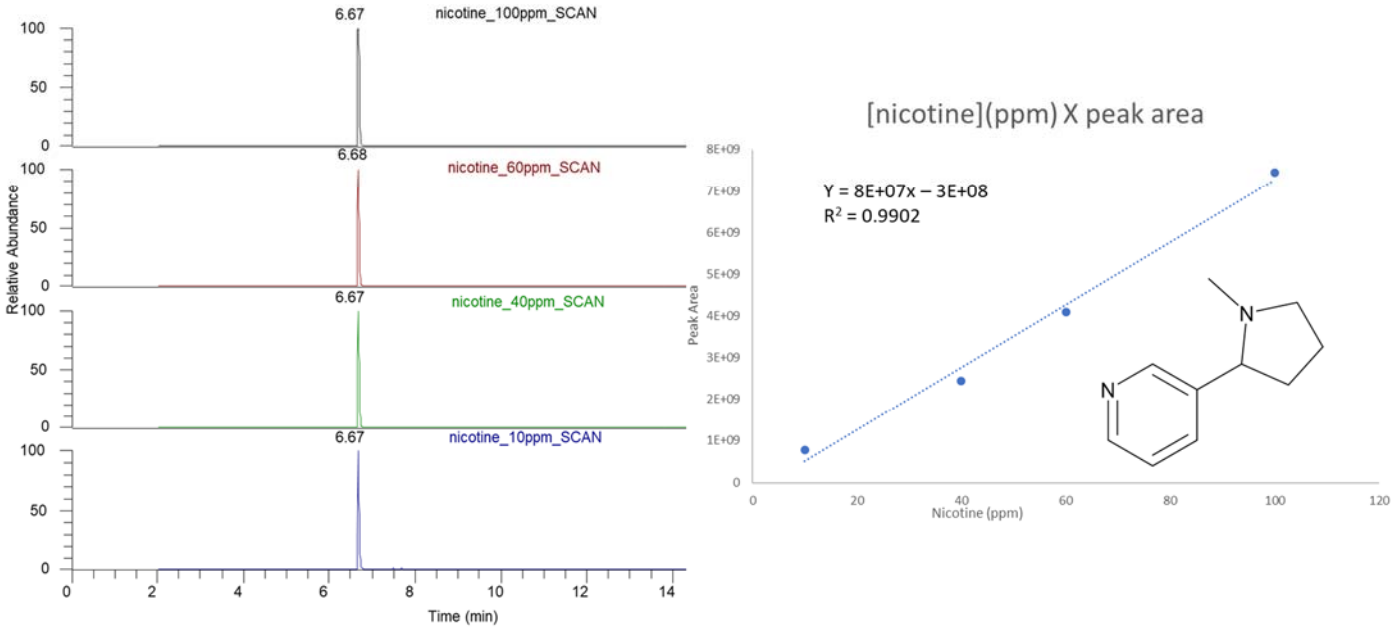


Figure 10 Chromatograms of 10, 40, 60, and 100 ppm nicotine standards (left) and Calibration Curve (right). Run on Thermo system in SIM mode with ions of interest of 83, 133, and 161 m/z.

Reproducing Extractions using the LLE Method Developed by Wilson

Before actual salamander secretion samples were extracted, a 100 mg/L nicotine standard dissolved in methanol was extracted using the LLE method developed by Wilson. Figure 11 contains the total ion chromatogram (TIC) of the extract obtained using the Agilent system, but there was no nicotine peak at the expected retention time of 7.5 minutes. The extracted ion chromatograms were examined at the characteristic m/z values for nicotine (84, 133, 161 as shown in Figure 10), and also lacked peaks, confirming loss of nicotine somewhere during the extraction. The other peaks in Figure 11 were not identified but were probably impurities typically observed using the Agilent system.

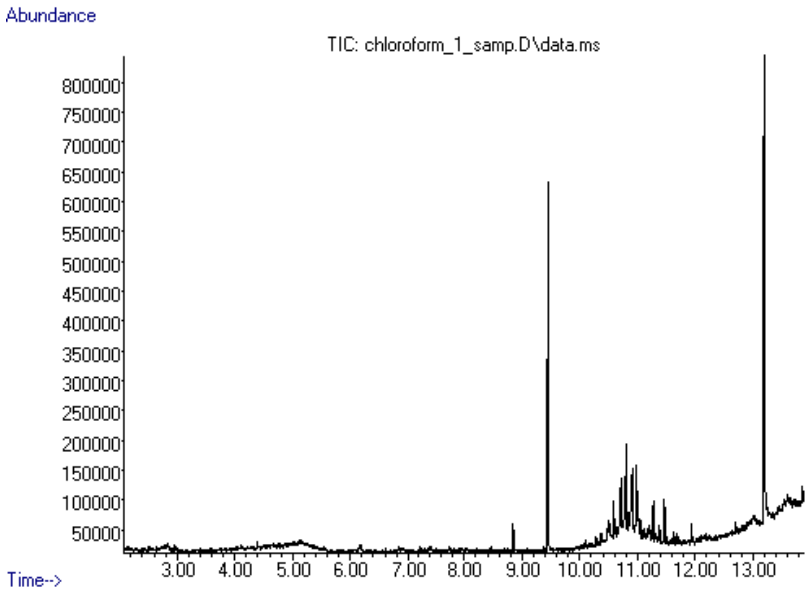


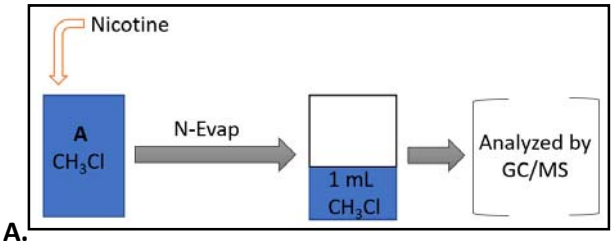
Figure 11 LLE of nicotine standard, extracted from a blank. Run on Agilent system with a 30-m VF-5ms column. No nicotine peak was observed at the expected retention time of 7.5 minutes.

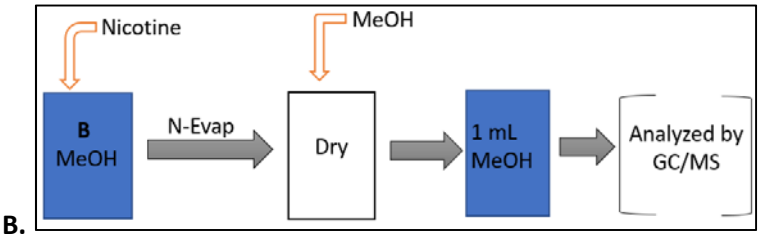
Nicotine could have been lost in several steps during the extraction. First, there was a lag time between extraction and analysis and the nicotine could have degraded during storage. Second, the concentrations of HCl (8 M) and ammonia (9 M) that were used during the extraction to protonate and deprotonate the nicotine were higher than those used the literature and perhaps were high enough to degrade the nicotine. Finally, nicotine could have been lost during the evaporation step. Loss during evaporation was explored next.

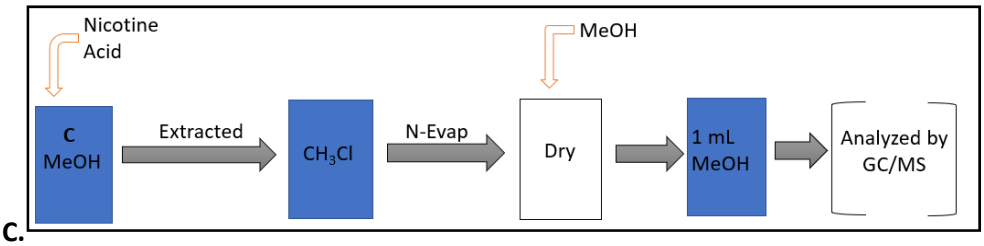
<u>Determining if Nicotine was lost during the Evaporation Step of the Extraction</u>

In the final step of the extraction, the chloroform layer is evaporated to dryness using nitrogen gas and the residue dissolved in 1 mL of methanol. The samples shown in Figure 12 were prepared to determine if nicotine was lost during evaporation. Samples A and B were not extracted at all; they were only evaporated. Sample A was a 1-mL nicotine standard dissolved in chloroform that was evaporated to a final volume of 1 mL of chloroform. Sample B was a 1-mL nicotine standard dissolved in methanol

that was evaporated to complete dryness, followed by dissolution in methanol. Samples C and D went through the entire extraction process. Sample C was evaporated to dryness in the final step, followed by dissolution in methanol. Sample C represents the way a sample would be evaporated in the final step of the extraction. Sample D was evaporated to 1 mL and left in chloroform. All samples were analyzed using the Agilent system.







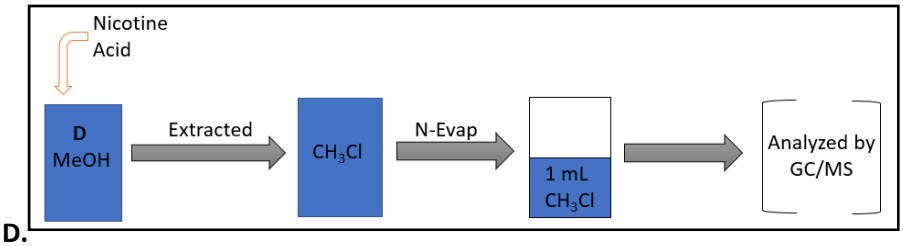


Figure 12 Evaporation study samples, Trial 1. Nicotine was dissolved in solvent and evaporated using nitration gas. **A:** 100 ppm of nicotine, dissolved in chloroform, evaporated down to 1 mL. **B:** 100 ppm of nicotine, dissolved in methanol, evaporated to dryness. **C:** 100 ppm of nicotine, extracted, in

chloroform, evaporated to dryness. **D:** 100 ppm of nicotine, extracted, in chloroform, evaporated to 1 mL.

The chromatograms for samples B and C that were evaporated to dryness contained peaks at 7.5 minutes (Figure 13), which were initially thought to be nicotine; however, the mass spectrum for this peak did not match that of nicotine (Figure 13). The peak at 7.5 minutes was likely a contaminant, but could not be identified from the mass spectrum. The chromatograms for samples A and D that were not evaporated to dryness also lacked nicotine peaks. Given that contamination was a recurring problem using the Agilent system, a simplified version of the experiment was repeated using the Thermo system.

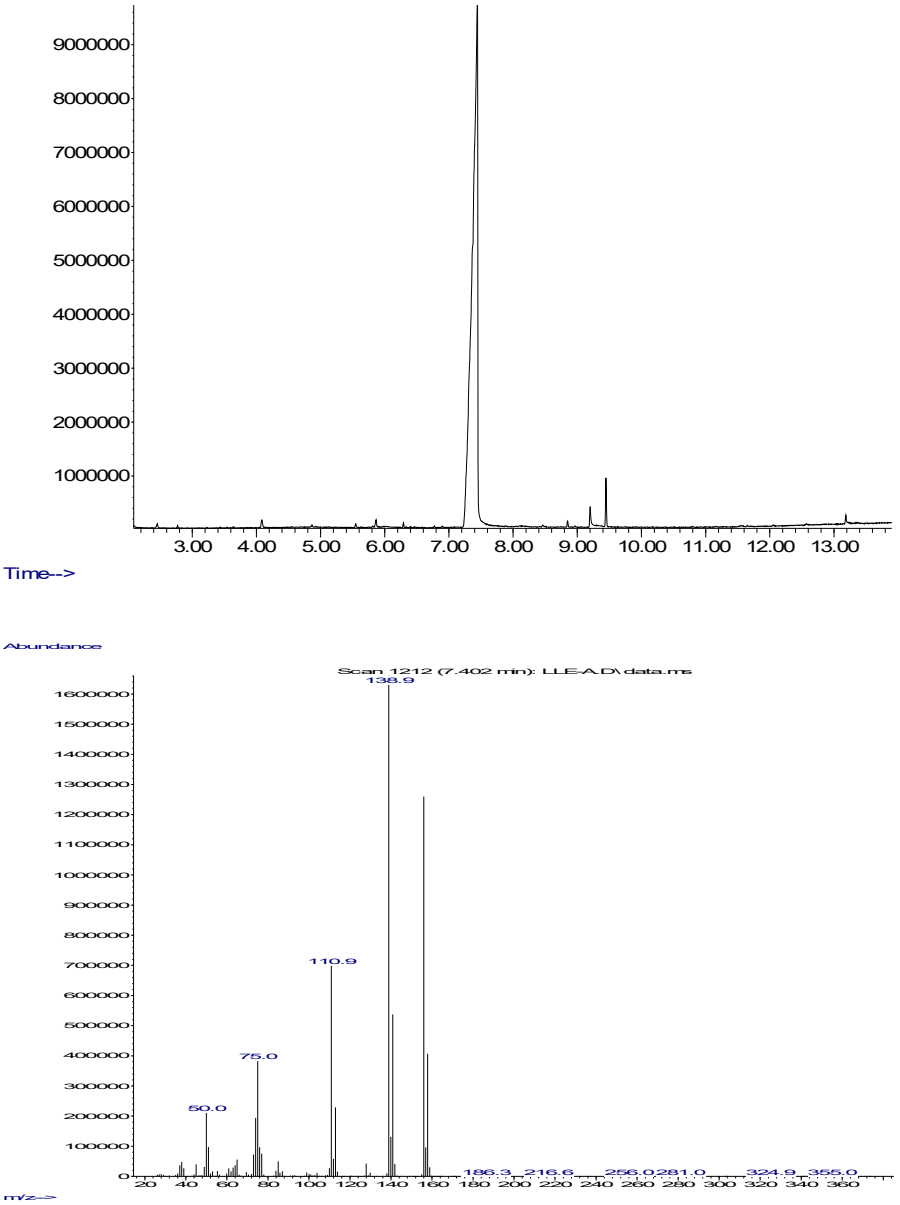


Figure 13 Chromatogram and Mass Spectrum for a 100 mg/L Nicotine Standard Evaporated to dryness (Sample A), 100 ppm Run on the Agilent system with a 30-m VF-5ms column. Mass spectrum is of the peak at approx. 7.5 min, originally believed to be nicotine, determined to be contaminant.

A 1-mL solution containing 100 mg/L nicotine in chloroform was evaporated to dryness and re-dissolved in 1 mL of methanol. After adding the methanol, however, a residue appeared at the bottom of the vial and would not completely dissolve (Figure 14). This residue was not observed in previous extractions. The sample was still tested using the Thermo system and found to contain 18 mg/L nicotine

using full scan mode and 43 mg/L using SIM mode, both much less than the expected concentration of 100 mg/L. One question remained: was the nicotine lost during evaporation, or, was it lost in the residue that wouldn't dissolve?

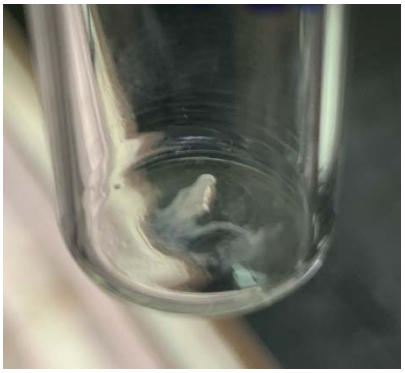


Figure 14: Residue from evaporation study, in methanol.

The samples shown in Figure 15 were prepared in order to answer this question. Sample A contained 100 mg/L nicotine in methanol, was evaporated to dryness, and re-dissolved in methanol. Sample B contained 100 mg/L nicotine in methanol but was evaporated to a final volume of 1 mL. Samples C, D, and E contained 100 mg/L nicotine in chloroform, were evaporated to dryness, then re-dissolved in methanol. The residue formed in samples C, D, and E, which all started in chloroform. The residue was vortexed but did not fully dissolve in methanol. The samples were analyzed using the Thermo system.

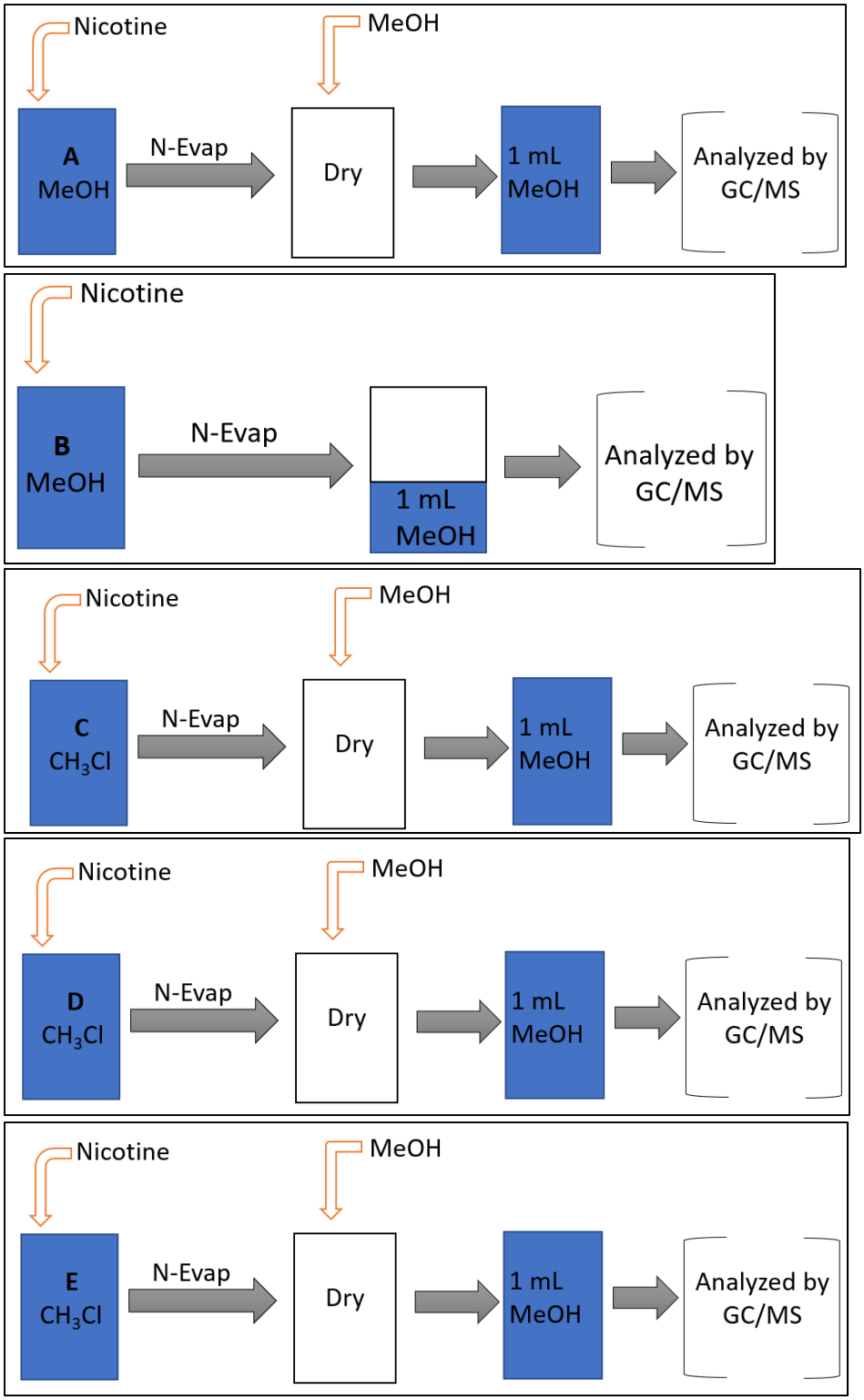
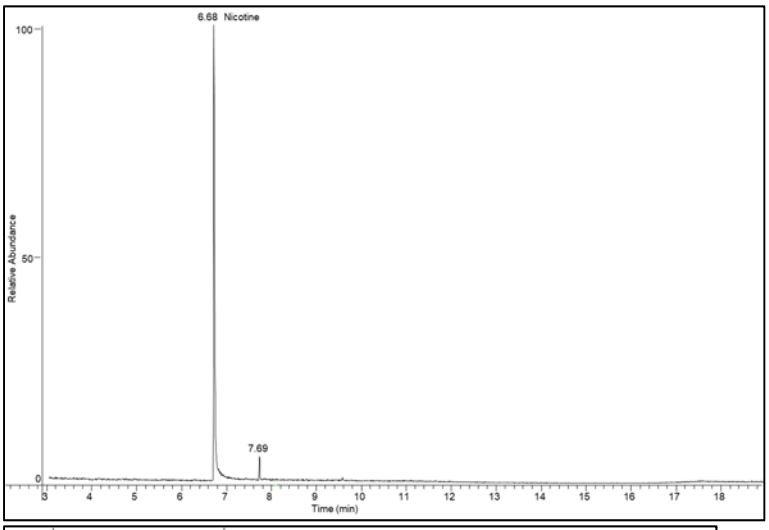


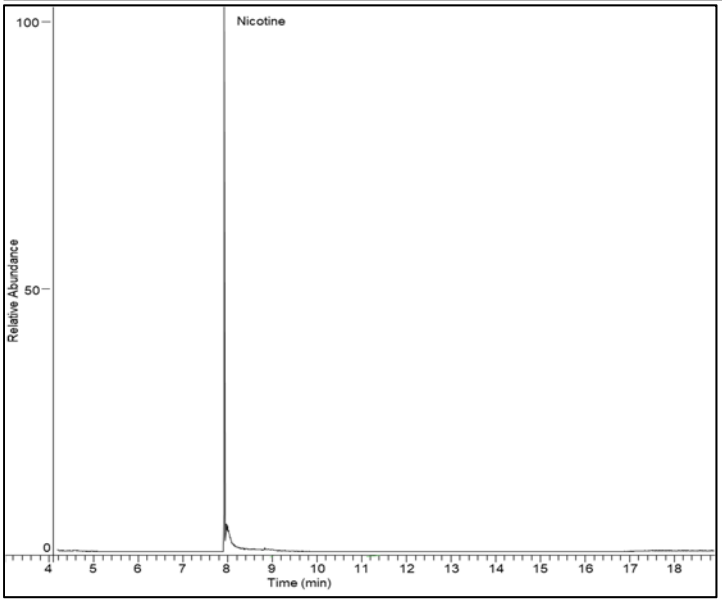
Figure 15 Evaporation study samples, Trial 3 Nicotine was dissolved in solvent and evaporated using nitrogen gas. **A:** 100 ppm of nicotine, dissolved in methanol, evaporated to dryness. **B:** 100 ppm of nicotine, dissolved in methanol, evaporated to 1 mL. **C, D, and E:** 100 ppm of nicotine, dissolved in chloroform, evaporated to dryness.

The Thermo system produced chromatograms that looked unusual, in that the signals were lower than expected and the nicotine peaks contained broad tails, as shown in Figure 15. Since this behavior had never been seen before, the Thermo system was troubleshooted, and it was eventually discovered that the ion source in the MS was not heating properly heating, leading to ions reaching the mass analyzer at slightly different times, hence the peak tailing. Ionization was probably incomplete, with some analyte not reaching the analyzer at all. Therefore, the peak areas from these chromatograms did not necessarily represent the amount of analyte present in the sample and were not used for quantification.

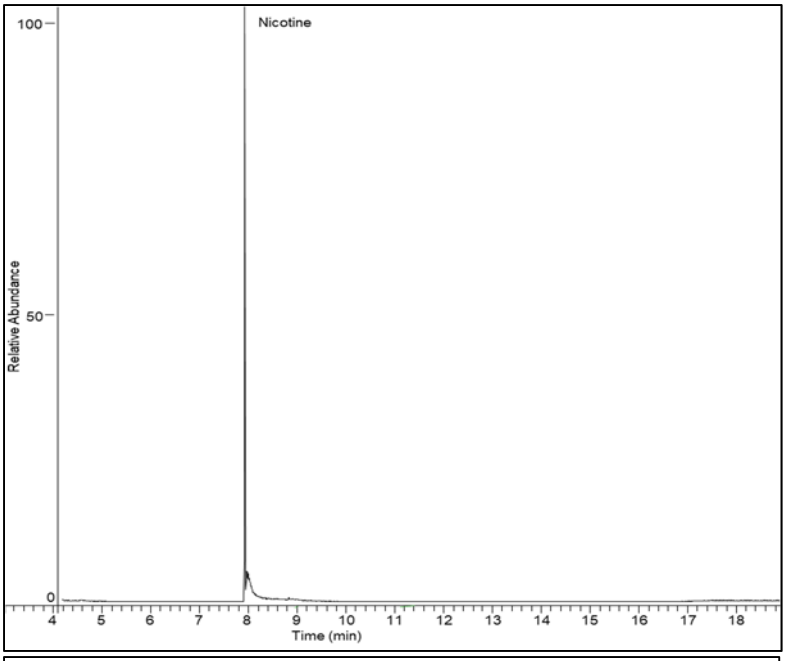
A.



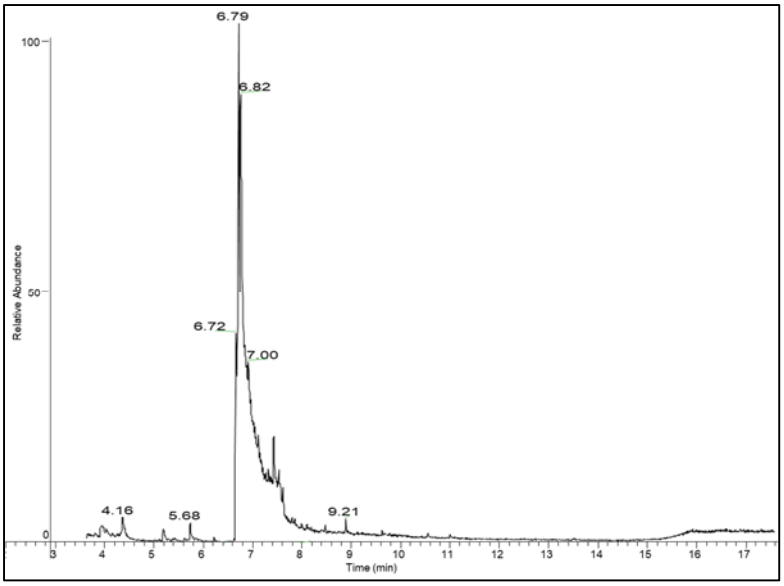
B.



C.


Nicotine
Relative Abundance
100
50
0
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time (min)

D.


6.79
6.82
6.72
7.00
4.16
5.68
9.21
Relative Abundance
100
50
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time (min)

E.

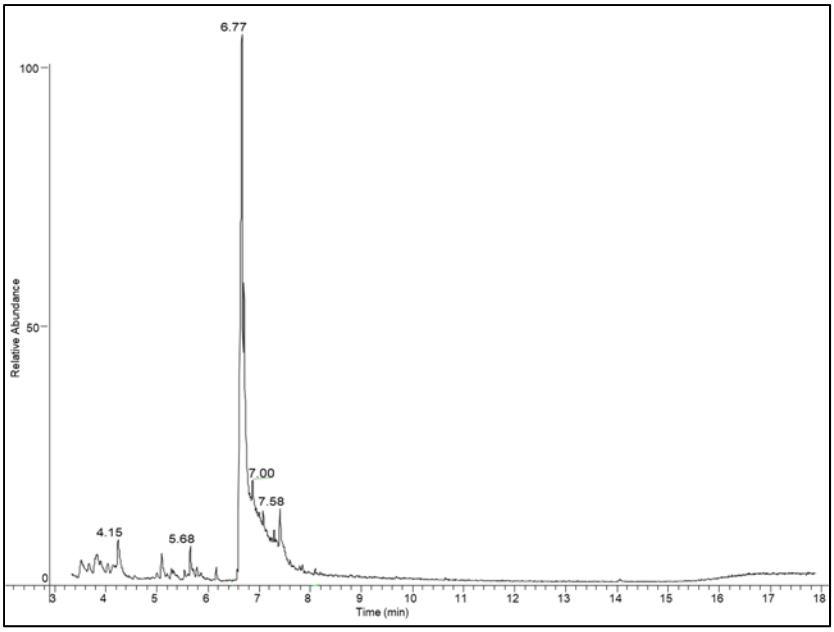
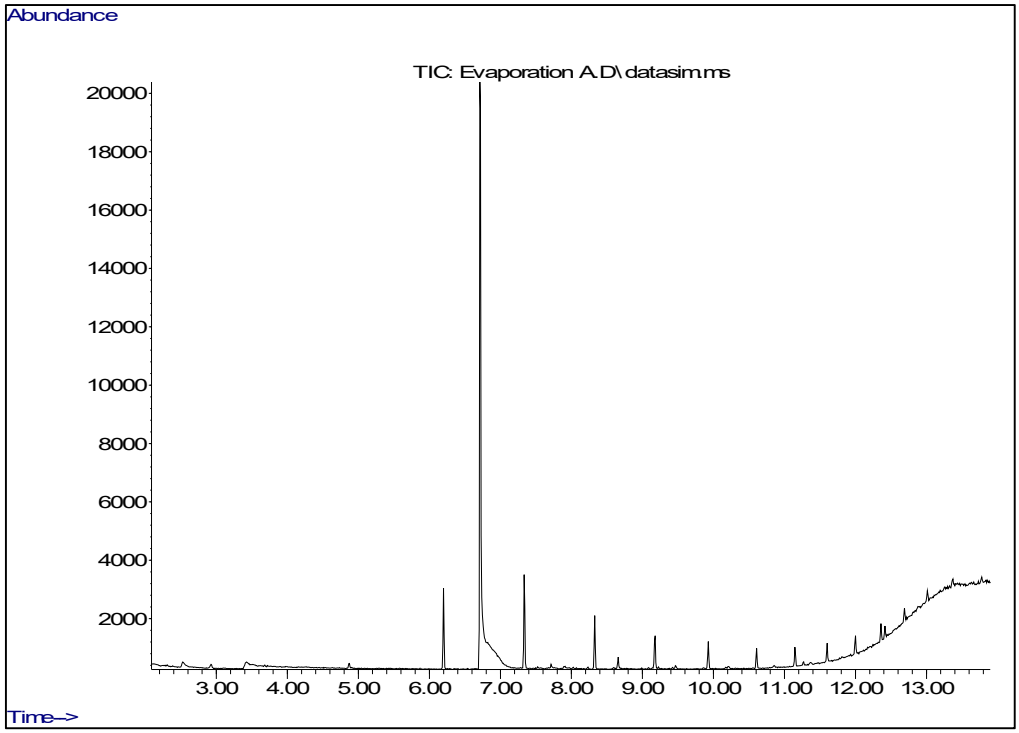


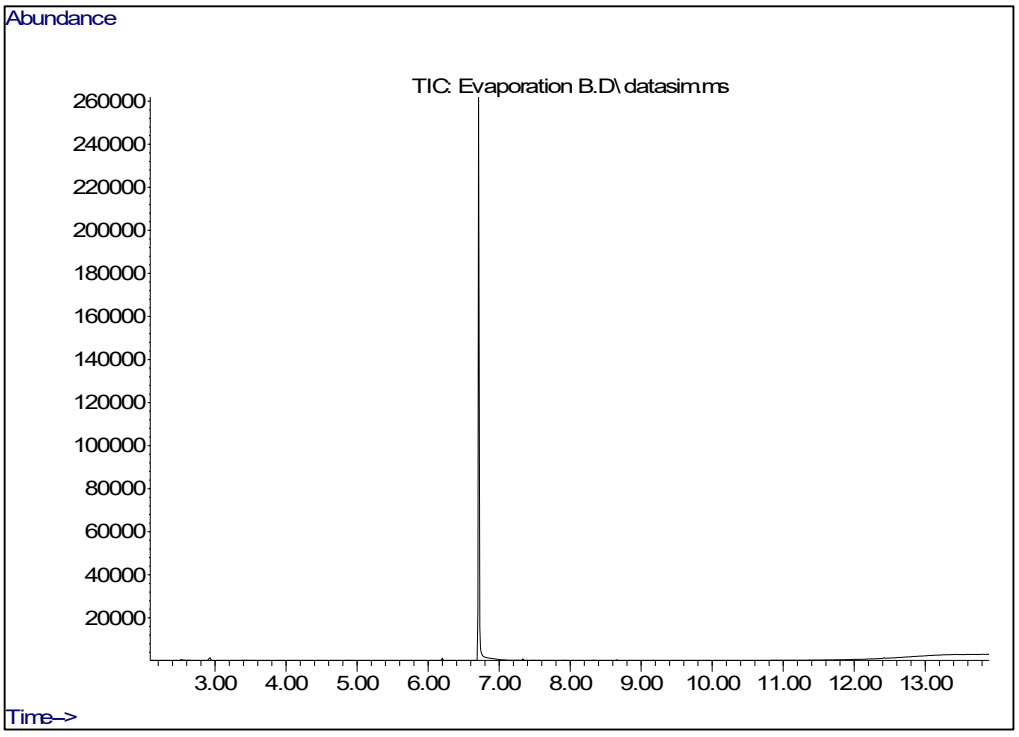
Figure 16 Chromatogram for a 100 mg/L Nicotine evaporation study samples, 100 ppm. Run on the Thermo system in SIM mode with ions of interest of 83, 133, and 161 m/z.

The samples were run on the Agilent system, and the chromatograms and peak areas can be seen in Figure 17 and Table 3, respectively. Sample A had the smallest peak area showing the most loss of nicotine during evaporation, with a 4 % recovery. Sample B also had a dramatic loss, with a 52 % recovery, although less than that of Sample A. Both samples A and B were dissolved in methanol, suggesting that samples dissolved in methanol are more susceptible to losing nicotine through evaporation. The three samples in chloroform, C, D, and E, all had peak areas relatively similar to the standard, with 64, 90, and 76 % recovery, respectively, showing that only a minimal amount of loss was occurring. These samples mimic the environment that the extracted samples would be in, so the loss in the extraction is most likely not occurring in the evaporation step. This experiment would be stronger with quantitative data, so the samples should be rerun, or remade and rerun on the Thermo system once the ion source issue has been resolved. Unfortunately, time constraints did not allow this to occur.

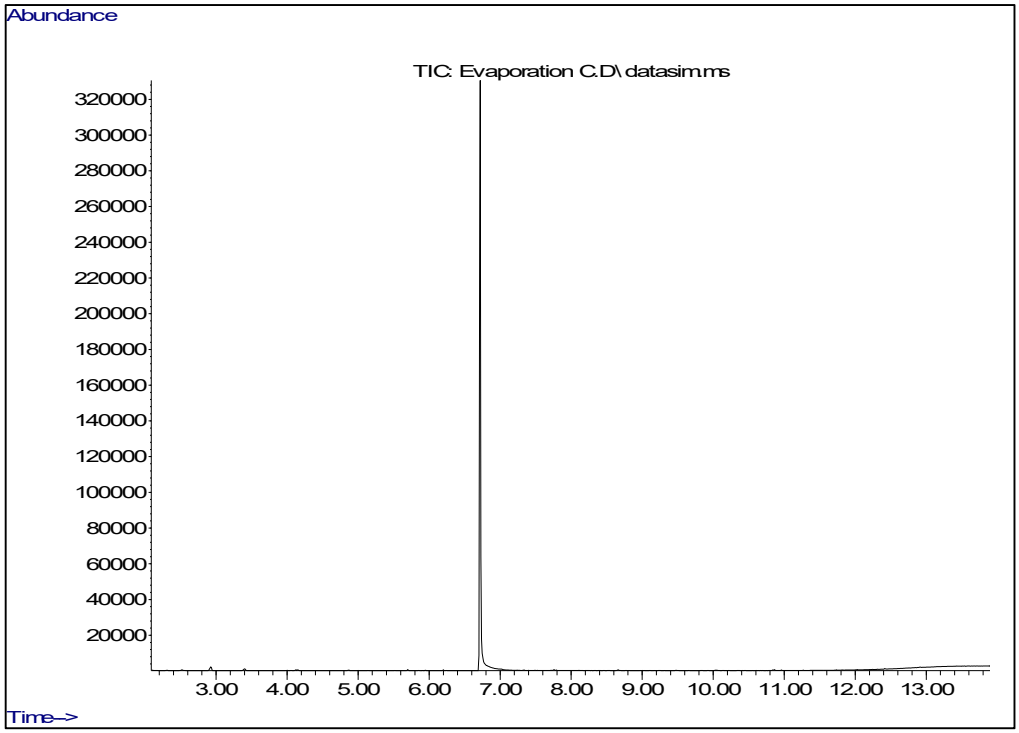
A.


Abundance
TIC: Evaporation A.D\datasim.ms
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Time-->

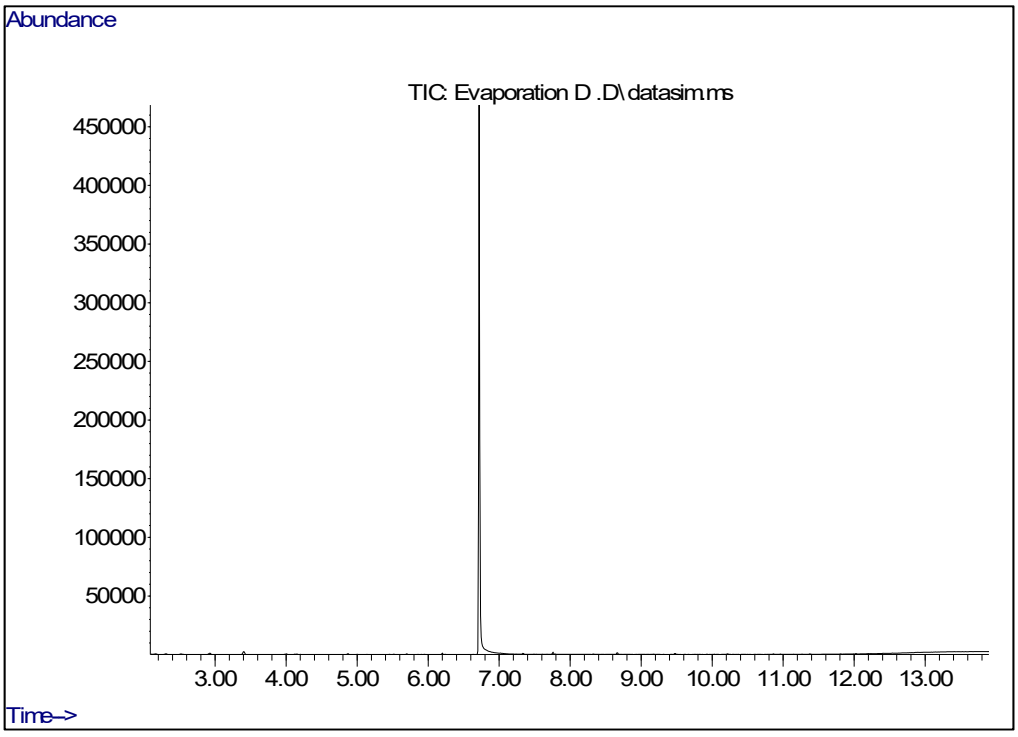
B.


Abundance
TIC: Evaporation B.D\datasim.ms
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Time-->

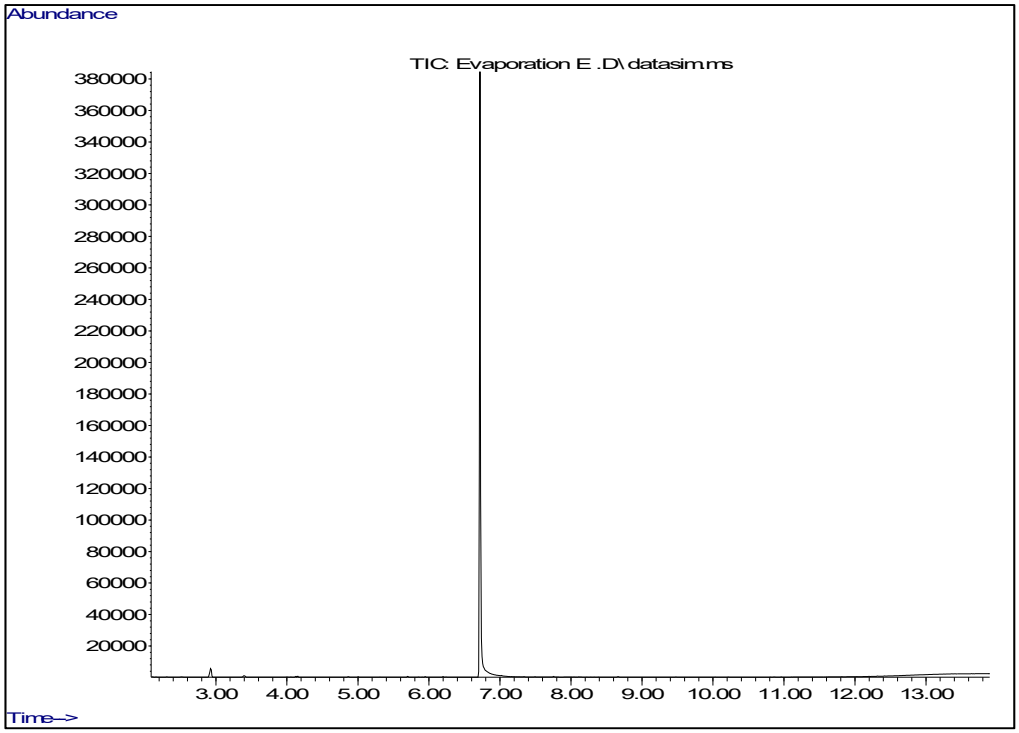
C.


Abundance
TIC: Evaporation C.D\datasim.ms
320000
300000
280000
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
Time->

D.


Abundance
TIC: Evaporation D .D\datasim.ms
450000
400000
350000
300000
250000
200000
150000
100000
50000
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
Time->

E.



F.

Figure 17 Chromatograms for 100 mg/L Nicotine evaporation study samples, 100 ppm. Run on Agilent system, with 30-m VF-5ms column. Run in SIM mode with ions of interest of 83, 133, and 161 m/z. **A.** in methanol, evaporated to dryness. **B.** in methanol, evaporated to 1 mL. **C.** in chloroform, evaporated to dryness. **D.** in chloroform, evaporated to dryness. **E.** in chloroform, evaporated to dryness. **F.** 100 ppm nicotine standard.

Table 3 Evaporation study samples peak areas Run on Agilent system, with 30-m VF-5ms column. Run in SIM mode with ions of interest of 83, 133, and 161 m/z. **A.** in methanol, evaporated to dryness. **B.** in methanol, evaporated to 1 mL. **C.** in chloroform, evaporated to dryness. **D.** in chloroform, evaporated to dryness. **E.** in chloroform, evaporated to dryness. Compared to 100 ppm nicotine standard.

Sample	Solvent	Evaporated	Peak Area	Percent Recovery
Nicotine Standard (100 ppm)			500,000	100 %
A	Methanol	To dryness	20,000	4 %
B	Methanol	To 1 mL	260,000	52 %
C	Chloroform	To dryness	320,000	64 %
D	Chloroform	To dryness	450,000	90 %
E	Chloroform	To dryness	380,000	76 %

Solid Phase Extraction

Initial SPE experiments were performed on a blank spiked with 100 mg/L surrogate using the Method A, shown in Table 2 in the experimental, where HCl was used to acidify the sample. Figure 18 shows the chromatogram of the final extract obtained using the Agilent system. A small peak can be seen at the expected retention time of 7.5 minutes, and the mass spectrum confirmed this peak to be nicotine. The other peaks in the chromatogram were not identified but believed to be column contamination usually observed on the Agilent system. The nicotine recovery was not determined, because the peak area was 50 times smaller than expected. Clearly, nicotine had been lost somewhere during SPE or storage of the extract. After the sample had been loaded onto the SPE tube, it could not be eluted immediately due to time constraints. The SPE tube was stored in the fridge for a few days with nicotine on it before elution, which wasn't ideal. Perhaps the concentrations of hydrochloric acid and ammonia that were used to protonate and deprotonate the nicotine during the loading and elution steps were not optimal. The presence of a nicotine peak—although smaller than desired—suggested that SPE may be a viable extraction method.

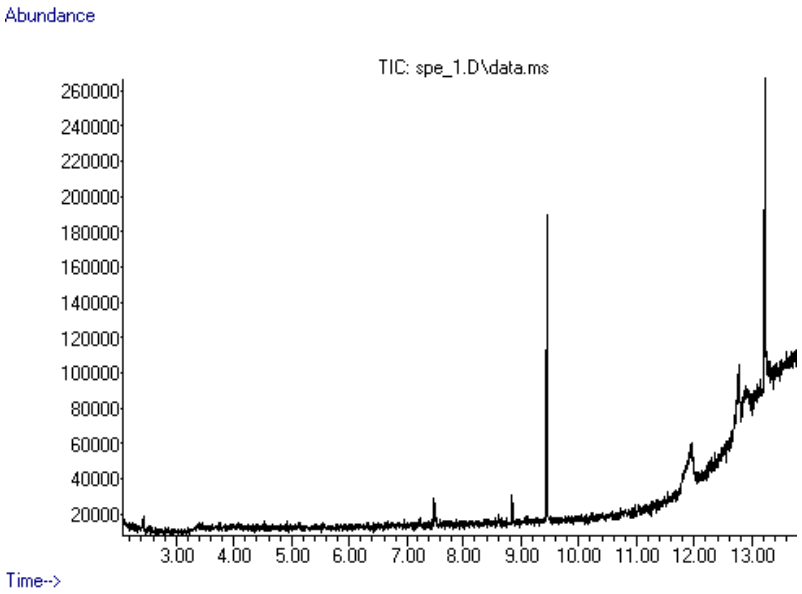


Figure 18 Chromatogram for a 100 mg/L Nicotine Standard extracted using SPE HCl was used to protonate the nicotine. See experimental section for more parameters. Run on Agilent system with a 30-m VF-5ms column. A small nicotine peak can be seen at the expected retention time of 7.5 minutes, but the peak area was 50x smaller than expected for 100 mg/L nicotine.

The SPE experiment was repeated, but this time, phosphate buffer was used to protonate the nicotine during the loading step (Method B, in Table 2 in the experimental). The pH of the phosphate buffer was 6.5, two units below the pK_a of nicotine, which should have been low enough to have protonated the nicotine yet high enough to have prevented degradation by a strong acid. These samples were run by HPLC instead of GC/MS, because they were eluted from the SPE bed using an ammonia-methanol solution that contained a small amount of water. As shown in Figure 19, a 100 ppm nicotine standard was also analyzed, and the chromatogram contained a large peak at approximately 7 minutes for nicotine. The SPE sample was run twice, and both chromatograms contained small peaks near the retention time of the standard but were much smaller than expected. Although recovery was poor, this SPE method could be a viable method with further development.

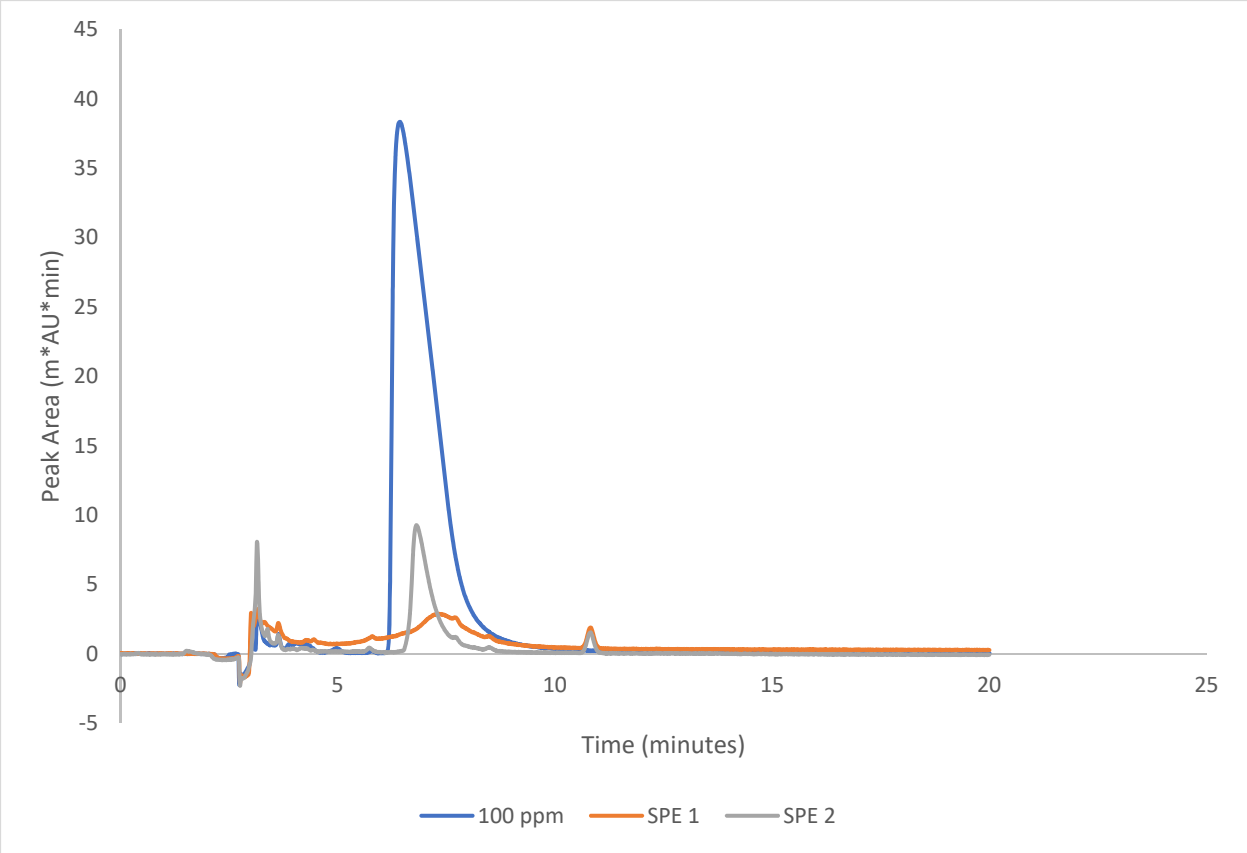


Figure 19 HPLC Chromatogram for a 100 mg/L Nicotine Standard extracted using SPE and a 100 ppm Nicotine Standard See experimental section for extraction parameters condition. Samples were analyzed on a U-3000 HPLC (Thermo) using a Luna C18 column (Phenomenex) with 50:50 ACN:water mobile phase at 0.7 mL/min. The blue line indicates the 100 ppm nicotine standard, the orange and grey lines indicate duplicate injections of the standard that was extracted using SPE. The duplicates (orange and grey) look so different, most likely due to a poor injection for SPE 1.

VI. Future Work

Sample Collection

Although benign for the salamander, the secretion collection method is not quantitative. The salamanders are kept wet throughout the process, and the amount of water in the secretion sample is unclear. The amount of secretion was not recorded and varied from salamander to salamander. The method of transferring the slime to vials was also problematic. The secretions were rinsed off the bag

and transferred into vials with methanol. Each vial had different initial amounts of secretion, water, and methanol. While this method is appropriate to use for qualitative work, if a more quantitative approach is taken, then the sample collection must be altered and optimized.

Previous papers have discussed a method of secretion collection where a cotton swab is pressed against a gland to stimulate secretion, and the secretions are then collected (Mebs). Another method electronically stimulates the salamanders to induce secretions before collection. A method developed by Von Byern (2017), describes placing salamanders on glass dishes covered in foil, making them visibly stressed, so that they would secrete and homogenously distribute the secretions on the foil. This collection method, or other potential collection methods could be tested, and may allow for more regulated amounts of secretions, and allow for the final concentration to be quantified.

Extraction Optimization

The efficiency of the extraction procedure must also be investigated. The evaporation study suggests that the evaporation step is not the area of loss in the extraction procedure. This means the remaining steps of the extraction should be examined for areas of loss. The SPE results suggests it may be a viable method, therefore future work should optimize the SPE method and examine the loss of nicotine. More nicotine standards should be extracted using SPE, and the percent recovery of the nicotine determined. If a large amount of loss still occurs, the steps involved in the SPE should be examined, and adjusted, to determine where the loss is occurring. Once the LLE and SPE methods are optimized, and the loss of nicotine is reduced, extractions should be performed on salamander samples, and analyzed for recovery of the standard, and appearance of peaks that may be alkaloids. If these peaks are visible, common alkaloid retention times can be used identify alkaloids, and the molecular weights can be used to predict mass to charge ratios that could appear in the mass spectra. This will potentially allow for the identification of alkaloids present in the salamander secretions.

VII. Conclusion

The *Plethodon yonahlossee,* a species of salamanders native to the Appalachian Mountains, displays bright colors and secretes when under attack. The coloring, as well as the secretions of the salamander, may indicate toxicity, and may have developed as a defense mechanism. By determining the chemical composition of the secretions, and potentially identifying the presence of alkaloids within the secretions, the toxicity of the salamander, and the purpose of the secretions can be evaluated. This project aims at developing an extraction method and using GC/MS to analyze the secretions. A liquid-liquid extraction method has been developed, and blanks spiked with a surrogate standard have been extracted and analyzed. Preliminary extraction results show loss occurring throughout the extraction method. The evaporation step has been studied, to examine if the loss of the standard occurred at this step. Preliminary results indicate this step is not where the loss is occurring. Solid phase extraction was also attempted and may be potentially viable. Two different GC systems were used to analyze extracts and obtain clean chromatograms. The limitations that come with this project are that the samples were collected three years ago, and that the method used is semi-quantitative. The ultimate goal is to develop and optimize extraction methods, and analyze the extractions with GC/MS.

VIII. References

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