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Determination of Cotinine Concentration in Urine by Liquid Chromatography Tandem Mass Spectrometry

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Abstract:

Tobacco use can cause lasting health conditions which can lead to death. Tobacco exposure comes from obvious sources like smoking or chewing tobacco, however these are not the only administration methods. Secondhand smoke exposure can affect even those who choose not to expose themselves to tobacco, which also opens them to the health issues that come along with tobacco use. Tobacco contains many different compounds, one of which is nicotine (Kataoka, 2009). Although nicotine is the most abundant compound in tobacco, it does not stay in the body for long periods of time. Nicotine metabolizes into cotinine which is more stable, therefore lasting longer in the body (Ahmad, 2020). A direct correlation has been established between cotinine concentration in body fluids and tobacco usage behavior (Doctor, 2004). The CDC National Health and Nutrition Examination Survey (NHANES) reports cotinine levels representative of the entire U.S. population and will be used as a comparison for this study (CDC, 2021). The purpose of this research is to investigate the secondhand smoke exposure in this population of non-smoking college students and compare to the levels of cotinine in the general non-smoking population to determine if college students are at greater risk of secondhand smoke exposure than the general population. This research was also done to investigate how different administration methods affect the cotinine levels in smokers. Cotinine was extracted from the urine samples using a solid phase extraction (SPE) method and then analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The average cotinine levels for smokers and non-smokers in this population are 47.02 ng/mL and 3.76 ng/mL, respectively. The non-smoking participants' cotinine levels could be from secondhand smoke exposure or from possible dietary cotinine. The smokers' samples showed cotinine levels that were similar for participants who smoked less per day than those who smoked more per day; this could be a result of dilution of the urine by the participant's liquid consumption. The method developed for this research successfully extracts and identifies cotinine from human urine. The analysis of more samples will allow for more insight into how the different administration methods affect cotinine levels.

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Introduction:

Tobacco use is a major public health issue, which can result in lasting health conditions or in many cases death (Kataoka, 2009). These health issues include renal, respiratory, and cardiovascular problems (Sanchez-Rodriguez, 2015) and extend to many cancers. The majority of cancer deaths worldwide can be attributed to tobacco smoke (Ahmad, 2020). In total there are over 4000 different compounds identified in tobacco, approximately 50 of these compounds are carcinogenic (Kataoka, 2009). All the aforementioned health issues are preventable by not using tobacco products, leading tobacco use to have one of the highest significance on preventable health issues, disability, and premature death (Sanchez-Rodriguez, 2015). These health concerns can be brought about by both firsthand and secondhand smoke exposure as well as other tobacco products like chewing tobacco (Henderson, 2020). By investigating the smoking behaviors of individuals, the potential risks for these health issues can be analyzed. Investigating various tobacco exposure pathways, like cigarettes, cigars, and chewing tobacco may aid in determining if there are differences associated with tobacco use based on the administration method used by the individual. Knowing the potential exposure to tobacco is especially important for individuals who do not smoke. Their exposure to tobacco within their environment could cost them their life.

As mentioned, there are many compounds present in tobacco products, alkaloids are among these compounds. Nicotine is the most abundant alkaloid and is readily absorbed into the body. It is metabolized into many different metabolites (Benowitz, 2009). About 70-80% of the nicotine that is absorbed is metabolized into the alkaloid compound, cotinine (Kataoka, 2009). Nicotine and cotinine are both biomarkers for tobacco exposure and are used to investigate smoking behavior as well as cases of secondhand smoke exposure (Doctor, 2004). Nicotine can be used for tobacco exposure identification; however, it is only found in minimal concentrations

within the body because it is readily metabolized into cotinine (Ahmad, 2020). Nicotine is metabolized into cotinine in a two-step process. The first is mediated by the enzyme CYP2A6 which produces an intermediate compound, nicotine-$\Delta^{1'(5')}$ -iminium ion which is in equilibrium with 5'-hydroxynicotine. The next step is catalyzed by cytoplasmic aldehyde oxidase to produce cotinine (Benowitz, 2009). Nicotine has a short half-life of only 2-3 hours (Ahmad, 2020). Because it has a longer half-life of 7-40 hours, cotinine will be identifiable for longer after exposure to tobacco (Ahmad, 2020). The longevity of cotinine in body fluids is the reason cotinine was chosen as the biomarker in this research.

Cotinine is an N-alkylpyrrolidine, a member of the class of compounds known as pyridines (PubChem, 2013). The structure can be seen in figure 1. The molecular weight of cotinine is 176.21 g/mol (PubChem, 2013). It can be found in human urine, saliva, and blood (Doctor, 2004). The elimination of cotinine in each of these body fluids is similar (Jarvis, 1988). Urine is the preferred matrix for tobacco exposure studies due to the higher concentration of cotinine when compared to saliva and blood (Doctor, 2004). The Center for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) had previously reported on cotinine levels in various populations based on age. This report is based on a sample size of approximately 1000 to 2000 individuals based on the years tested and the population demographics needed to statistically represent the entire population of the U.S. It is used to provide statistical information about the health of adults and children in the U.S. NHANES will be used as a comparison for the results found in this study (CDC, 2021).

The detection of nicotine and cotinine as biomarkers for tobacco use could be affected by the presence of dietary nicotine. Nicotine can be found in various plants, vegetables, and teas (Davis, 1991). The Solanaceae family of plants like eggplant, tomato, and green pepper have

been found to contain nicotine (Davis, 1991). There is no cotinine found in these sources, however the metabolization of nicotine happens inside the body as it does with tobacco use, therefore it is found in the body after consumption of these foods. The response of nicotine and cotinine seen in individuals who consumed these types of foods are similar to the types of responses seen in cases of second-hand smoke exposure, low concentrations of both nicotine and cotinine (Davis, 1991). A study done on dietary nicotine estimated cotinine levels to be 0.6 to 6.2 ng/mL based on the results found for the nicotine concentrations (Davis, 1991). This poses an issue in analysis of tobacco usage especially in second-hand smoke exposure cases.

Although cotinine can be found in various bodily fluids, this research focuses on urine. Since urine contains many different compounds, the analytes of interest should be isolated to reduce interference and background noise thereby improving the limit of detection. Isolation of target analytes can be accomplished by an extraction method, such as solid phase extraction (SPE). With SPE, a liquid sample is passed through a cartridge containing a stationary phase with an affinity for the analyte of interest (cotinine), allowing other compounds to flow through the cartridge. The cartridge is then flushed with a mobile phase that has a higher affinity for the target analyte removing it from the stationary phase for analysis. SPE is fast, high quality, and cost effective for sample preparation (Doctor, 2004). Previous research conducted in 2003 involved the analysis of the urine of tobacco harvesters using SPE and liquid chromatography (LC). This particular study used a Drug Test-1 column from Analchem Ltd. for the sample cleanup (Doctor, 2004). The column is a chemically modified silica column, which contains cation exchange and hydrophobic and polar characteristics (Doctor, 2004). Since cotinine and nicotine are both polar compounds, they will be selectively retained on the column (Doctor,

2004). A small amount of solvent with a high affinity for the analytes of interest was used for the elution of the compounds from the column (Doctor, 2004).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an analytical technique that combines separation of chemical species in a mixture with their subsequent identification. Liquid chromatography is ideal for exploring these compounds because of its ability to separate based on properties of the compounds, such as polarity, size, or structure. In the study previously mentioned, a C18 column was used to separate the compounds of interest (Doctor, 2004). Having an extra carbonyl group on the cotinine molecule makes it more polar than the nicotine molecule, causing nicotine to elute from the non-polar C18 column at a faster rate. Mass spectrometry is a detection method that is ideal for identification of many compounds. Compounds are identified based on the fragmentation patterns of the compounds which are unique due to bond types, chemical composition, and structure. In a study conducted in 2011, an atmospheric pressure chemical ionization source with a triple quadrupole mass spectrometer was used. This instrument had the sensitivity to detect low concentrations, as low as 0.05 ng/mL, like those found with secondhand smoke exposure (Jacob, 2011). LC-MS/MS is ideal because the separated compounds can be identified on their own without interference from other compounds.

Materials and Methods:

Samples

The urine samples used in this research were obtained from 18 out of 23 community colleges across Virginia as part of a Virginia Department of Health Institutional Review Board approved study. There were over 700 samples for testing. Each participant had the option to sign

a waiver for their sample to be used and retained for future testing along with filling out a questionnaire asking about demographic information and general lifestyle questions about drinking habits, diet, hobbies, occupation, education as well as smoking habits. Because these samples were collected from community colleges, the age range is somewhat limited. The majority of the participants were within 18 to 35 years old. The samples were collected from 2016-2019 and were stored in -80°C.

Reagents and Standards

Ultra-pure water (18.3MΩ) (Barnstead E-pure DCLS deionized water supply), HPLC grade methanol (Honeywell), HPLC grade acetonitrile (Honeywell) were all used throughout this research. Other chemicals used were certified ACS grade ammonium acetate (Fisher Scientific), 99% ammonium bicarbonate (Acros Organics), certified ACS grade acetic acid (Plus), certified ACS grade ammonium hydroxide (Fisher Scientific). The standards used in this research were 100 ppm cotinine in methanol (Supelco) and 100 ppm cotinine-D3 in methanol (Supelco). Certified drug free human urine (Utak) was used to create the standards in this research.

Equipment

There are various pieces of equipment used in this research. Eppendorf digital pipettes of size ranges 5-100 μL and 100-1000 μL, were used throughout the research to aid in the precise measurements needed for the urine and other solutions. An Orion Star A211 pH meter was used in the preparation of the ammonium acetate buffer. A Biotage TurboVap 96 was used in the dry down step of the extraction. To mix the samples in the plates and to seal the plates for analysis on the instrument a Maximix vortex mixer and an Axygen PlateMax semi-automatic plate sealer

were used, respectively. The extraction process was carried out on a Biotage Extrahera robotic extraction platform which aided in reducing the time for the extraction.

Working Standards and Controls

The cotinine calibration standards ranged from 0.05 ng/mL to 100 ng/mL. The amount of cotinine found in smokers and nonsmokers ranges from 0.3 to approximately 1800 ng/mL as reported by the NHANES report (CDC, 2021). The range chosen was determined based on the extraction plate's capability. The extraction plates were not robust enough for the range to be increased past 100 ng/mL. How the samples with concentrations higher than this range were dealt with will be addressed later in this paper. The dilution scheme for the calibration standards preparation can be seen in table 1. Three quality control (QC) standards were used in this research: a low at 0.5 ng/mL, a medium at 5 ng/mL, and a high at 50 ng/mL. These span the entire range of the calibration standards to ensure the high and low end of the calibration is accurate. Both the calibration standards and the quality control standards were run with every plate of samples. The dilution scheme for these standards can be seen in table 2. The stock cotinine-D3 (deuterated cotinine) was diluted to 100 ng/mL for use as the internal standard (Li). This was done because it has an almost identical structure to cotinine and eluted in the analysis time on the instrument, but because of the difference in molecular weight it can be differentiated from cotinine.

Sample preparation

For preparation of the urine samples, 500 µL of sample and 50 µL of internal standard, 100 ng/mL deuterated cotinine, were aliquoted into the appropriate well of a 96-well sample plate (Phenomenex, elution/collection plate). The plate was vortexed for at least 30 seconds to

thoroughly mix the contents. Each plate contained a set of calibration standards in the concentrations mentioned previously along with the three quality control standards. Batches of samples were bracketed with the QC standards.

Extraction procedure

The extraction was carried out on a robotic extraction platform. The samples were diluted with 0.5 mL of 20 mM ammonium acetate buffer pH 4 to ensure the target analyte, cotinine, has the correct charge to interact with the SPE plate (Watson, 2018). In this extraction, a 96-well Phenomenex Strata X-C 33 μm Polymeric Strong cation plate was used (Li, 2018). The SPE plate was conditioned with 1 mL methanol then equilibrated with 1 mL 20 mM ammonium acetate buffer. The buffer was used to ensure the plate sorbent is charged and the analyte will be retained on it (Watson, 2018). The diluted samples were then loaded onto the 96-well SPE plate. The first wash was 2 mL of the 20 mM ammonium acetate buffer (Li, 2018). This wash was to remove all other unwanted materials present in the sample that could add noise in the analysis while keeping the pH the same to retain cotinine on the plate. The second wash was 2 mL 30% methanol in water (Li, 2018). This wash also helped to remove other materials in the sample and did not change the pH ensuring cotinine was retained on the plate (Watson, 2018). The samples were eluted using 2 rinses of 1.5% ammonium hydroxide in methanol. This basic solution changed the pH and therefore the ionization of the sorbent which released cotinine from the plate (Watson, 2018). The eluted samples were then dried down using nitrogen gas at 55°C in a TurboVap and were reconstituted with 100 μL 10:90 acetonitrile/20 mM ammonium bicarbonate to match the mobile phase for analysis (Li, 2018). The samples were then transferred to a 96-well V-bottomed microelution plate (Abgene). The plate was sealed using a plate sealer.

Instrumentation

The LC used in this research was an Agilent 1290 Infinity II with a Phenominex Gemini 3 μm NX-C18 110 Å column. The mobile phase in this research was a 20 mM ammonium bicarbonate (solution A) and 100% acetonitrile (solvent B) (Li). A gradient mobile phase was used to decrease the analysis time on the instrument and to ensure separation of the cotinine from other compounds that were not removed during the extraction procedure. The gradient scheme of the mobile phase is shown in table 3. The flow rate was set at 0.5 ml/min and temperature of the column compartment was set at 25 °C (Li, 2018). The injection volume was 10 μL (Li, 2018). The MS used in this research was a Sciex 4500 QTRAP with nano-spray ionization source in Multiple Reaction Monitoring (MRM) mode to select the ions of interest.

Results and Discussion:

Extraction

Although LC is a chromatographic method, an extraction is necessary to isolate and concentrate the cotinine and reduce the possibility of interferences from other compounds that elute at the same time as cotinine. There was one limitation found with this extraction method during this research. When there was a high cotinine concentration, the plate failed to extract all the cotinine. Due to the size of the plate and the amount of sorbent present only so much cotinine can be retained on the plate. This issue arose in the early stages of the calibration process when the higher concentrations of standards did not produce linear results in the calibration curve. The high end of the curve was lowered to accommodate this. This also means that any samples analyzed that have a concentration outside the calibration would have to be diluted and reanalyzed, then their true concentration would need to be back calculated. Due to the limited

time frame of this research, this was not done to any of the highly concentrated samples. Further testing of these samples could still be done.

Validation

The validation procedures followed were the parameters set by the Virginia Division of Consolidated Laboratory Services Verification/Validation for Analytical Methods and Instrumentation Standard Operating Procedures (SOP). This SOP is based on various guidelines by the US Food and Drug Administration and the International Organization for Standardization (DCLS, 2019).

During the beginning stages of developing the method, a chart for each QC standard was created. QC standards were analyzed with each calibration run. Twelve of these runs were used to create the QC charts using the average for each QC sample and their standard deviations. All the QC standards analyzed along with the samples for this research were put in the chart to check that they were within 3 standard deviations of the mean. The QC chart for the 50 ng/mL QC standard can be seen in figure 3. This ensured that the analysis was carried out correctly. If all the QC results were within three standard deviations, they were not significantly different from the initial QC runs that were conducted during the calibration runs. This means the instrument was running in the correct way to produce results that were acceptable for the QC standards. This ensured that any results from the sample are accurate and not due to an error in the instrument.

Each of the samples went through a verification process of 3 different checks to ensure that the response seen was truly from cotinine in the sample and not due to noise or any interferences. This included using a cotinine response for quantitation and a cotinine confirmation response. Cotinine (quantitation response) was confirmed and quantified based on

the fragmentation of the molecular ion, 176.892 m/z, and the second most abundant ion, 79.994 m/z. The confirmation response was identified based on the fragmentation of the molecular ion, 176.892 m/z, and the third most abundant ion, 98.022 m/z. The internal standard, deuterated cotinine, was confirmed and quantified based on the fragmentation of the molecular ion, 180.043 m/z, and the second most abundant ion, 79.938 m/z. The identification ions can be seen in table 4.

The verification process included a check of the area ratio, a check of the signal-to-noise ratio, and a check of the calculated concentrations. The ratio of the area under the peaks between cotinine quantitation response and the cotinine confirmation response were determined for the calibration standards and the QC standards. This resulted in a range that the sample area ratios should fall to ensure that the peak seen is truly cotinine and not an interference. It is unlikely that any compound other than cotinine would have area ratios within the range that is seen with the cotinine quantitation and cotinine confirmation responses. The next check was to consider the signal-to-noise ratio. The ratio must be above 10, as dictated by the DCLS Verification/Validation for Analytical Methods and Instrumentation SOP, to ensure the response seen is not from noise. The last check was to consider the calculated concentrations of the cotinine and the cotinine confirmation. These must have a percent difference less than 10% which ensures these responses are for cotinine as they have similar concentrations. The samples must pass these three checks. If one does not pass it means that the response could be from an interference and not actually from the target analyte, cotinine.

Chromatography

Figure 4 shows the total ion chromatogram of the sample extracts. The run time was 5 minutes with cotinine eluting at 1.28 minutes. The internal standard also eluted at 1.28 minutes

because it is deuterated cotinine so the interactions it had within the column are the same as cotinine making it elute at the same time.

Calibration curve

The calibration curves were linear over the 0.05-100 ng/mL range as seen in figure 5. All linear regressions had a correlation coefficient (r^2) greater than 0.9900. With a high correlation coefficient, the results have a good fit in the linear regression. The calculated concentrations for participant samples will be accurate to the true concentration present in the sample.

Sample Results

In total, 275 samples were run through this method. Of these 275 samples, 34 were from individuals who smoked, 226 were from individuals who do not smoke (but 2 use chewing tobacco), and 19 of these individuals did not complete the questions about their smoking habits. The data for the 19 samples with missing information about their smoking habits was ignored in the analysis of smoker and non-smoker groups.

The samples were given one of 4 different determinations based on the results from the verification checks of the area ratios, signal-to-noise ratios, and the calculated concentrations, which included present, not present, lower than calibration, and greater than calibration. Present was given to any samples that passed all the verification requirements, meaning the response seen is truly from cotinine. Samples that did not pass the verification requirements were categorized as not present meaning the response seen is not from cotinine and from an interference. Lower than calibration or greater than the calibration was given to any samples that passed all the validation requirements, but the calculated concentration was outside the calibration range used, below 0.05 ng/mL or above 100 ng/mL respectively, therefore cotinine is

present however the true concentration is unknown. The results of this determination is shown in table 5.

The average cotinine concentration in the smoker samples was 47.02 ng/mL. They ranged from 9.89 to 100 ng/mL with a standard deviation of 26.63. These results were from just the samples that had cotinine present, therefore these results are not representative of this entire population of smoking samples. The distribution of these samples is shown in figure 6. The samples from smokers that were greater than the calibration range were not included as their true concentration was not found. The CDC reported the cotinine concentration in human urine of smokers for ages 20-49 years as 1,410 ng/mL for 2013-14 and 1,160 ng/ml for 2015-16 as part of the NHANES report (CDC, 2021). These are much higher than the results seen for these samples. This however makes sense because the calibration range is not large enough to accommodate the larger concentrations because the extraction method is not robust enough to extract high concentrations of cotinine, higher than 100 ng/mL, without dilution of the sample before analysis. The smoker samples breakdown into 4 different administration categories, cigarettes, cigars, e-cigarettes, chewing tobacco. There were a few participants that used multiple administration methods which broke down into 2 categories, cigarettes/e-cigarettes and cigarettes/e-cigarettes/cigars. The number of samples for each category can be seen in table 6. For the cigar, e-cigarette, chewing tobacco, cigarettes/e-cigarettes, and cigarettes/e-cigarettes/cigars categories had 3, 3, 2, 1, 2 samples respectively. Due to the small sample sizes for these categories few conclusions can be drawn about the administration methods' effect on cotinine levels. The cigarettes category had the highest number of samples analyzed at 21 with a range of 1-20 cigarettes smoked per day (with a standard deviation of 5.76). There were only 3 samples in this category within the calibration range and the rest had a cotinine concentration

greater than 100 ng/mL. These samples were from participants who smoked 2, 4, and 9 cigarettes per day and they had cotinine concentrations of 41 ng/mL, 58.6 ng/mL, and 58.5 ng/mL, respectively. There were 2 samples that both reported the number smoked per day as 4 however, one's cotinine concentration was within the calibration range at 58.6 ng/mL and the other had a cotinine concentration greater than 100 ng/mL. This result was also seen with 2 samples that reported 2 cigarettes smoked per day, one with a cotinine concentration of 41 ng/mL and the other with a cotinine concentration greater than 100 ng/mL. This could be from the dilution of urine due to the liquid consumption of the individual which could misrepresent the cotinine concentration from what was metabolized from the nicotine absorbed into the body, which can be corrected by normalizing the cotinine concentration using creatinine. This was not done due to limited time constraints of this research. More samples of participants who smoked should be analyzed to see more patterns within and between the different administration sources.

The average cotinine concentration in the non-smoker samples is 3.76 ng/mL. This includes all the non-smoking samples that had cotinine present and all the non-smoking samples where cotinine was not present. The range for these samples is 0.0191 to 89.4 ng/mL with a standard deviation of 10.88. The distribution of these samples can be seen in figure 7. One of the samples from the non-smokers category had a concentration of 89.4 ng/mL. This value is much higher than all the other samples in this group. Removing this sample does not change the average for the non-smoker samples significantly as the mean would fall from 3.76 ng/mL to 3.13 ng/mL with a new standard deviation of 8.035. The CDC NHANES reported the cotinine concentration in human urine of non-smokers for ages 20-49 years as 0.588 ng/ml for 2013-14 and 0.603 ng/ml for 2015-16 (CDC, 2021). These results are lower than the results from the samples in this research. This could be due to sample size as the number of samples analyzed in

this research is much less than in the NHANES report. There could also be discrepancies of the answers to the questionnaire and the true habits of the participants, but there is no way to know if the answers that were given are untruthful. Due to the samples' collection for a different study, questions about the participants' secondhand smoke exposure were not asked as part of the questionnaire. Because of this, no conclusion about the effect of secondhand smoke can be drawn from these samples. These results could also be seen from dietary nicotine consumption which has been metabolized to cotinine. Diet was included on the questionnaire however, it only included when the participants ate and how many times per week they ate at each meal, therefore conclusions about the results having been from dietary nicotine cannot be made.

Conclusion:

Concentrations of cotinine were analyzed from samples collected from community college students in Virginia for insight into the effects of tobacco exposure. The average cotinine concentration in this group of smokers for the samples where the cotinine concentration was in the calibration range is 47.02 ng/mL, however many of the smokers samples have a concentration greater than 100 ng/mL. The samples that were outside the calibration range should be reanalyzed after an initial sample dilution so the true cotinine concentration can be found. Though the different administration sources were analyzed, more samples are needed to make any full conclusions about their various effects on cotinine levels. These samples should go through a correction to account for the dilution of the urine due to liquid consumption. This could be done as a creatinine correction, which is an amino acid that is released into the plasma at a constant rate then is processes by the kidneys (Uchino, 2010). The average cotinine concentration in this group of non-smokers is 3.76 ng/mL. The smoking habits of this population of community college students somewhat differs from the CDCs report. The smoker population

has a lower average than the CDC, however because many of the samples in this research were not used for this group, the comparison of these groups is not accurate. The non-smoker population has a greater average than the CDC's report. The difference in these values could be due to a couple possibilities: untruthful answers on the questionnaire, exposure to second-hand smoke, or dietary nicotine. Because one of the explanations for the high cotinine levels is secondhand smoke exposure, these students could also be at risk for the health effects that are related to tobacco use. Though some conclusions can be made from the information given, there is information missing from the questionnaire that would have been beneficial to this research: such as, if the participants live with a smoker, if the smoker in the home smokes inside the residence, and if the participants regularly eat any of the foods that contain dietary nicotine. This would aid in making conclusions about how this population is being affected by secondhand smoke.

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Figures and Charts:

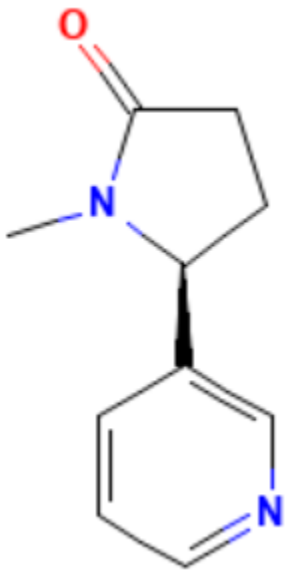

Figure 1: Structure of cotinine (PubChem, 2013)

Table 1: Dilution scheme for cotinine calibration standards.

	Std 0	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**
Flask volume (mL)	50	50	50	50	50	50
Volume of calibration standard (µL)	0	0.025	0.05	0.5	5.0	50
Final Concentration (ppb)	0	0.05	0.1	1	10	100

Table 2: Cotinine quality control standards dilution scheme.

	QC Low	**QC Medium**	**QC High**
Flask Volume (mL)	50	50	50
Volume of QC Std (µL)	0.25	2.5	25
Final Concentration (ppb)	0.5	5	50

Table 3: Gradient of mobile phase, 20mM ammonium bicarbonate and acetonitrile (Li, 2018).

Time (min)	Acetonitrile (%)
0	10
3	75
3.1	10
5	10

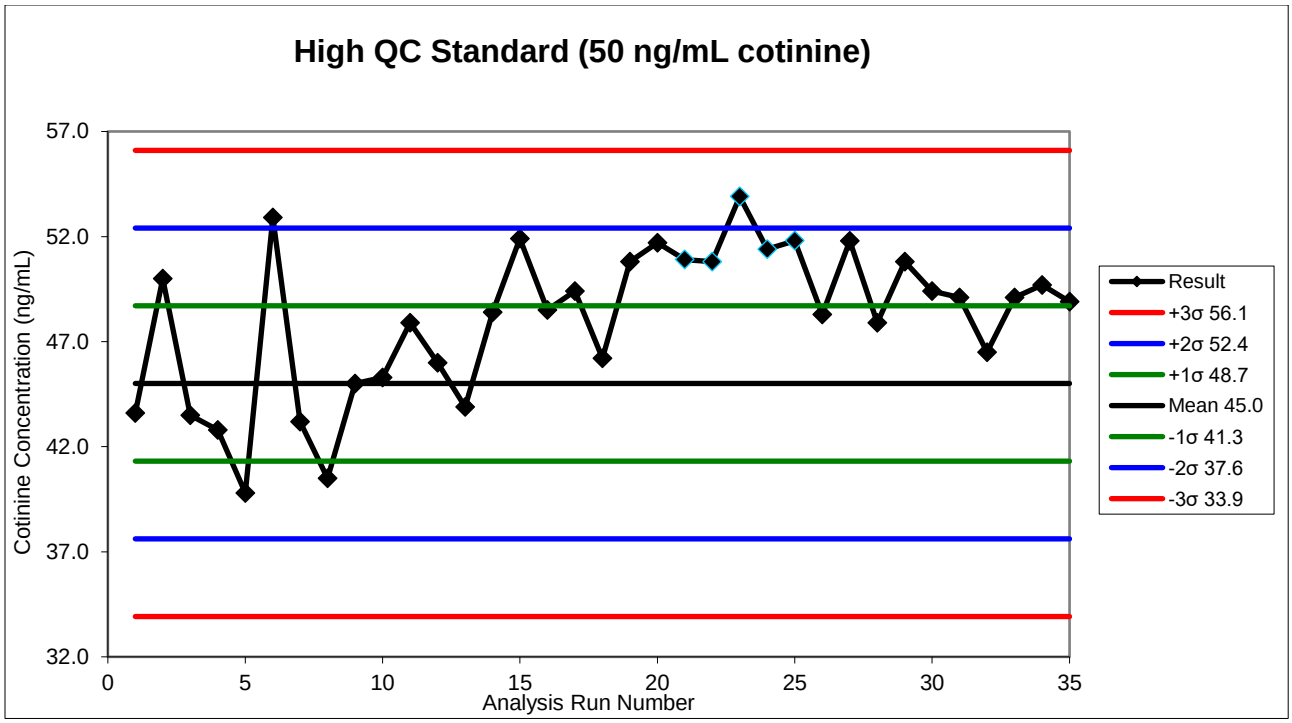

Figure 3: QC chart for the high QC standard at 50 ng/mL cotinine. All QC standards analyzed were within 3 standard deviations of the average cotinine concentrations detected.

Table 4: Cotinine quantitation, cotinine confirmation, and internal standard identification ions.

	Product ion (m/z)	Confirmation ion (m/z)
Cotinine quantitation	176.892	79.994
Cotinine confirmation	176.892	98.022
Internal standard	180.043	79.938

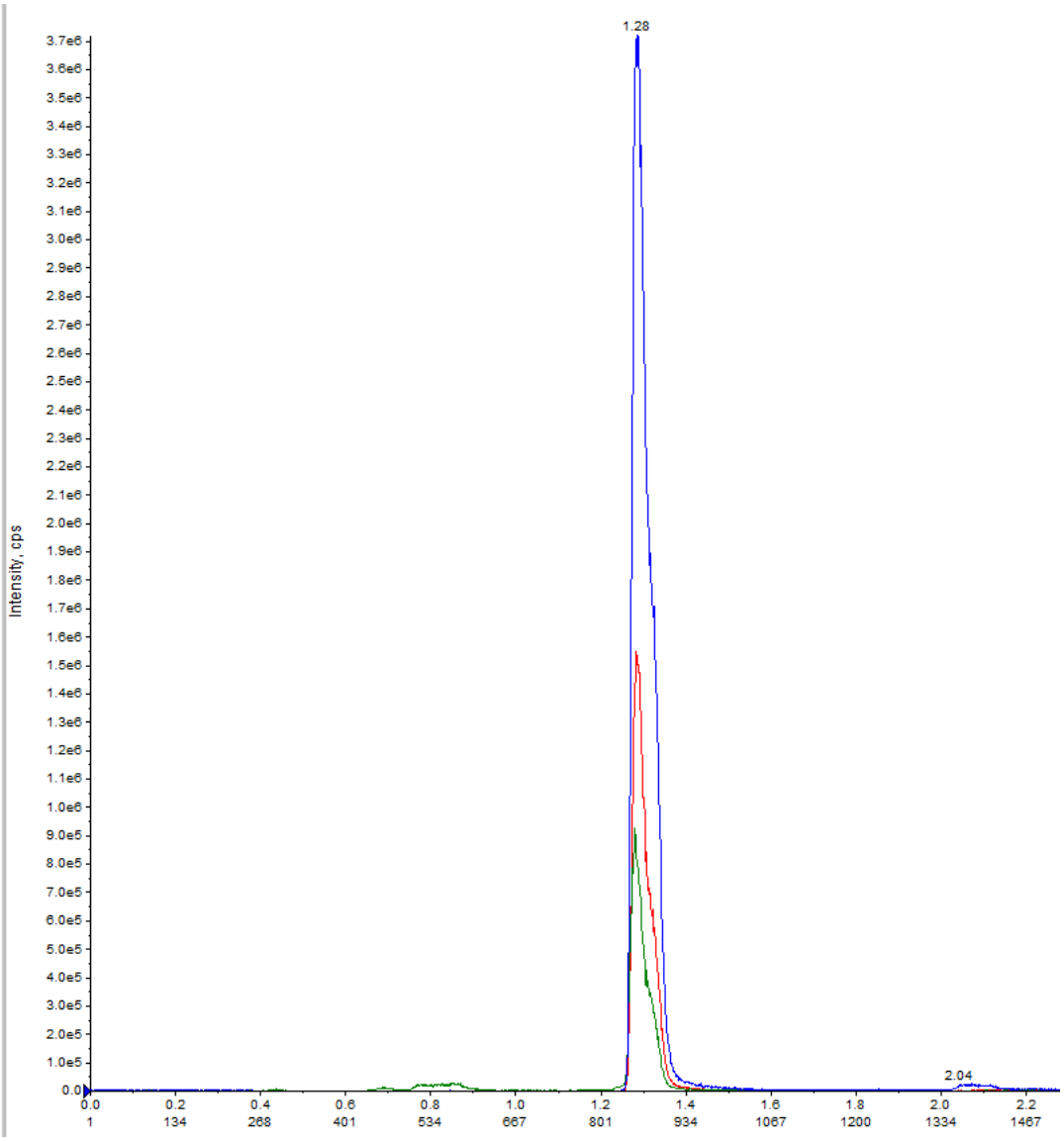

Figure 4: Total ion chromatogram for the cotinine quantitation (blue), cotinine confirmation (red), and the deuterated cotinine internal standard (green). The chromatogram was cropped, no peaks show on the chromatogram past cropped area.

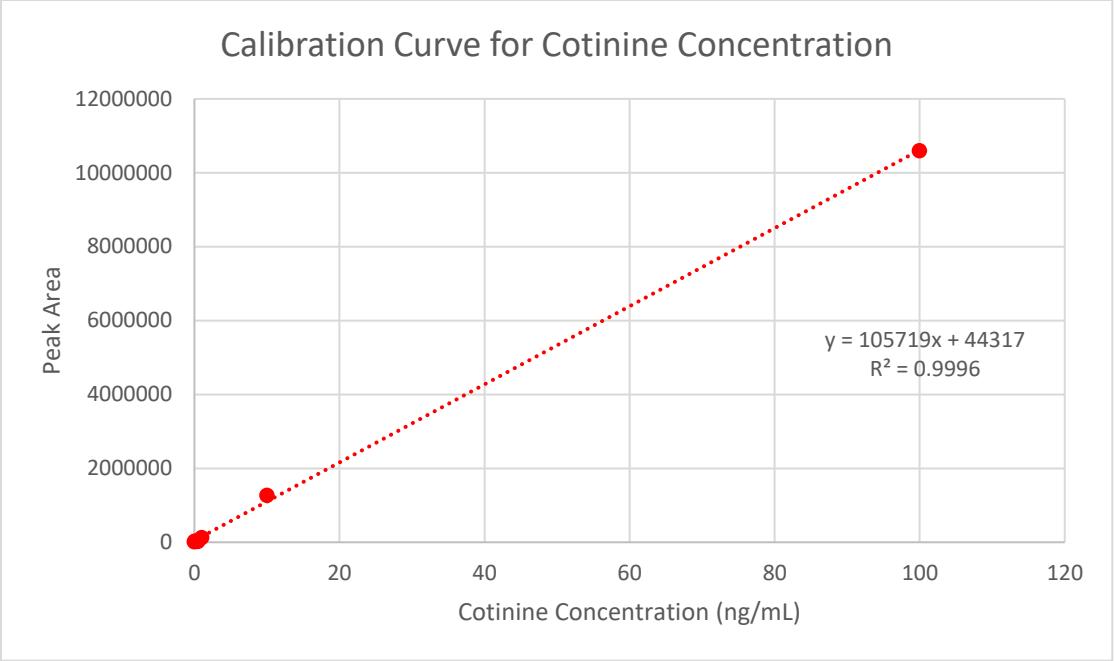

Figure 5: Calibration curve for cotinine concentrations 0.05-100 ng/mL.

Table 5: Summary of determinations of the samples analyzed.

	Smoking	Non-Smoking	Missing information
Present	10	133	12
Not present	0	64	5
Lower than calibration	1	20	0
Greater than calibration	24*	4	2

*Two of these participants do not smoke but do use chewing tobacco

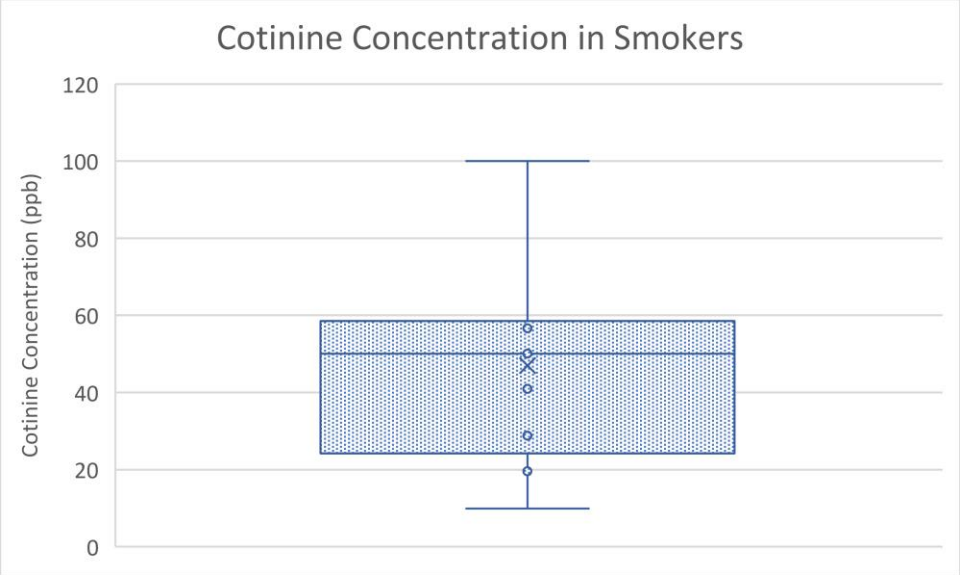

Figure 6: Distribution of cotinine concentration in smokers from 0-100 ng/mL. The bottom part of the box represents the lower quartile of cotinine concentration for the smoking category while the top part of the box represents the upper quartile. The center line is the median, 50.1 ng/mL.

Table 6: Breakdown of administration methods for the samples in the smoking category

	Cigarettes	Cigars	E-Cigarettes	Chewing Tobacco	Cigarettes and E-Cigarettes	Cigarettes, E-Cigarettes, and Cigars
Number of samples	21	3	3	2	1	2

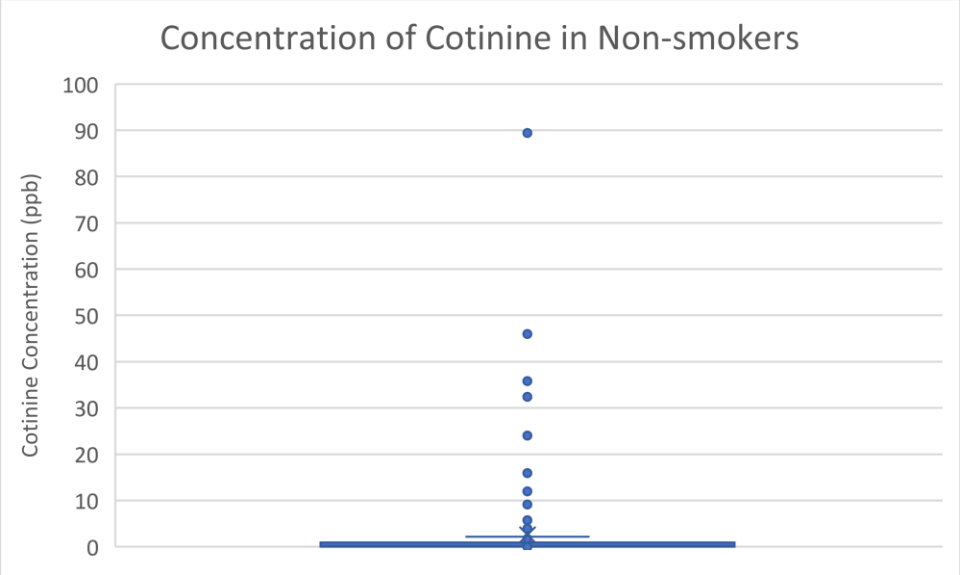

Figure 7: Distribution of cotinine concentration in non-smokers from 0-100 ng/mL. The bottom part of the box represents the lower quartile of cotinine concentration for the non-smoking category while the top part of the box represents the upper quartile. The center line is the median, 0.41 ng/mL. Many of the samples for this population are not within the lower and upper quartile making it difficult to view the box. The points above the box are the samples that are outside this range.

Vita:

Allison Beckett is from Shrewsbury, Massachusetts. She received a Bachelor of Science in Forensic Chemistry from Western New England University in 2020 and graduated summa cum laude. In August, she will graduate from Virginia Commonwealth University with a Master of Science in Forensic Science with a concentration in Forensic Chemistry/Trace. During her time at both institutions, she worked as a teaching assistant, aiding students with labs and grading lab reports.