Development of a Method to Detect Nitazenes in Seized Drug Samples

By

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Abstract

Due to fentanyl and its analogs being scheduled drugs, they are more difficult to acquire, so illicit traffickers turn toward novel synthetic opioids. Nitazenes are an emerging class of synthetic opioids that are steadily increasing in popularity. In 2019, the Midwestern United States saw the first overdose cases involving isotonitazene and the number of cases, and variety of analogs have only increased. Nitazenes are extremely potent, on par with fentanyl and its analogs, about 1000x more potent than morphine. This makes them dangerous and the need for their identification is greater than ever. However, they constantly get overlooked due to minimal information about their usage. In this thesis, the best color tests for identification of nitazenes were determined, the best extraction technique was determined, and a gas chromatography-mass spectrometry (GCMS) method was optimized for the identification of nitazene citrate, metonitazene, isotonitazene, protonitazene, etonitazene, and etonitazepyne.

Out of Marquis, nitroprusside, Mecke, Froehde, van Urk, Wagner, cobalt thiocyanate/ stannous chloride, ferric chloride, Dille-Koppanyi, and Liebermann's, the best color tests were Marquis, Libermann's and Wagner. Out of base extraction, pH 9 buffer extraction, and solvent dissolution with methanol, the best extraction determined for all of the analogs was base extraction with sodium hydroxide and toluene. A GCMS method was then developed for the analogs modified from the Sacramento District Attorney's Lab of Forensic Services GCMS method. The method's parameters were: 15-minute run time, 30:1 split ratio, injection volume 1 µL, and a temperature ramp rate of 20°C/min with a hold at 280°C for nine minutes. When run with this method, all of the nitazenes had good separation and abundance, allowing them to be added to the internal GCMS libraries. In addition to using the GCMS to characterize the nitazenes, Fourier transform infrared spectroscopy (FTIR) and TruNarc technology were also

evaluated. Isotonitazene, nitazene citrate, and protonitazene were run on the FTIR and added to the internal library of that instrument. Metonitazene and etonitazepyne were not run on FTIR because they were in solutions of methanol and etonitazene was not run because there was not enough of the standard left to produce a usable spectrum. All analog standards were run on the TruNarc, however due to the amounts of etonitazene and protonitazene and the composition of the instruments' internal library, only isotonitazene was able to be identified using this method. Finally, counterfeit pills found in the casework of the criminalists in the Controlled Substances section were analyzed for nitazenes and found not to contain any nitazene analogs evaluated in this study. As a result of this research, the Sacramento County District Attorney's Lab of Forensic Services now has a working method to identify nitazenes in their casework.

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Chapter 1: Literature Review

Synthetic opioids have rapidly increased in popularity in the last 15 years. Beginning with the reemergence of fentanyl on the drug scene in 2008, novel synthetic opioids are becoming the main cause of drug overdoses in the United States[1]. Synthetic opioids, such as fentanyl, are extremely potent and it often can take less than 1 milligram (mg) for a fatal overdose to occur[1]. Opioids are not available for commercial use and are considered suitable for limited medical purposes. Fentanyl itself is 50-100 times more potent than morphine[2]. As fentanyl and its derivatives increased in popularity from 2008 to 2018, they have also become increasingly controlled, eventually being classified as a Schedule 2 drug by the Drug Enforcement Administration (DEA). Due to stricter regulations of already existing synthetic opioids, users began to turn to novel psychoactive substances (NPS) and moved away from fentanyl-based analogs and towards less restricted and cheaper substances.

During the COVID-19 pandemic, the use of NPS skyrocketed, as it was much easier to synthesize chemical precursors and hospitals were overrun with COVID cases, so their capability to detect and report NPS use reduced drastically[3]. Forensic toxicology laboratories did not predict the uptick in synthetic opioid use and were not prepared when new synthetic opioids became available on the market. The use of new synthetic opioids began to increase in 2013, as seen in Figure 1[4]. The DEA was not able to schedule the new substances quickly so addiction and overdose rates increased drastically. During the pandemic, synthetic opioids were the most abused class of NPS and resulted in the highest number of deaths of all the drug classes[3]. In 2020, it was recorded that 80% of drug overdoses were from NPS, increasing the concern of government drug enforcement agencies[5].

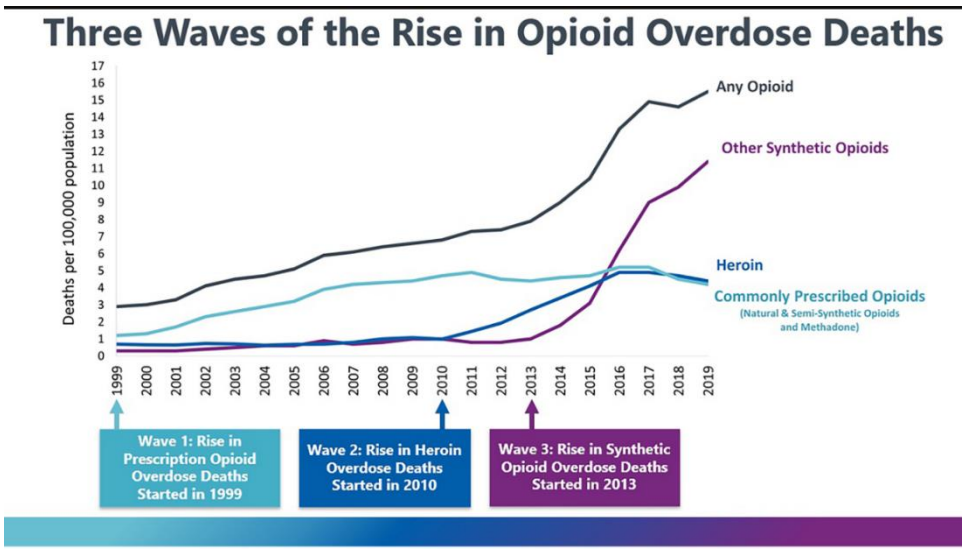


Figure 1 *Opioid deaths over the three waves of the opioid epidemic[4].*

One class of these new synthetic opioids is 2-benzylbenzimidazoles, known as nitazenes. Originally developed in 1957 as an alternative to morphine, nitazenes are structurally dissimilar to the opium poppy based opiates as well as fentanyl and its analogues[6]. Although a Swiss pharmaceutical company originally created the narcotic analgesic nitazenes to assist with pain, they were never approved to be sold on the market due to their extremely high potency[6,7]. Initially synthesized by Swiss chemists A. Hunger, J. Kebrle, A. Rossi, and K. Hoffman of CIBA Pharmaceuticals, 2-benzylbenzimidazoles were found to create a strong analgesic affect when a nitro group was in the 5 position[6]. They were encouraged because they believed that they had found a safe alternative to morphine and methadone for sedating people[6]. They detailed the methods they used to synthesize these new compounds as well as how the different substituents they attached had different affects[6]. In the years following their initial synthesis, Hunger and Hoffman patented their new creation in 1960[7]. In 1975, Alexander Shulgin, a pharmacologist in California, warned that etonitazene and clonitazene have a high abuse potential and no redeeming medical utility[8]. Nitazenes do not appear again in literature until 1999 when the DEA

reported that they had encountered a clandestine lab that was illegally synthesizing etonitazene[9]. The DEA also cited a case of etonitazene overdose found in Moscow, Russia in the same report[9].

There is record of nitazenes being found in the United States before 2019, though they were few and far between. A case, as recorded by Deseret News, in Utah in 2003, saw a man caught synthesizing etonitazene at his workplace, Morton Thiokol[10]. The Deseret article then goes on to say that the DEA stated that this was the first time nitazenes of any sort had been illegally manufactured in the United States, and that the only other known labs to have synthesized them (besides the original Swiss CIBA) were in 1987 in Germany and 1998 in Russia[10]. The investigators at the time concluded that he was only creating the drug for his own use and that it would not become popular due to the specific chemistry knowledge needed to produce it[10]. The only other mention of nitazenes before 2019 is in 2009 when Hamilton Morris gave an interview to Vice about etonitazene. He discussed etonitazene specifically because until 2019, that was the only nitazene analog that was in use. He cited the 2003 case concerning the Utah man that synthesized etonitazene for his own use[11]. Morris also makes sure to inform the readers etonitazene is typically only found in labs studying addiction and is rarely sold on the streets[11]. Though Morris is not a scientist, he describes etonitazene as having 1,500 times the potency of morphine with the stimulant effects of ecstasy[11]. After 2009, there was no interest in nitazenes, as fentanyl had taken the world by storm, so nitazenes fell by the wayside until 2019.

In 2019, a greater amount of nitazene analogs began being introduced onto the synthetic drug scene. Examples of the chemical structures of nitazenes analogs are provided in Figure 2.

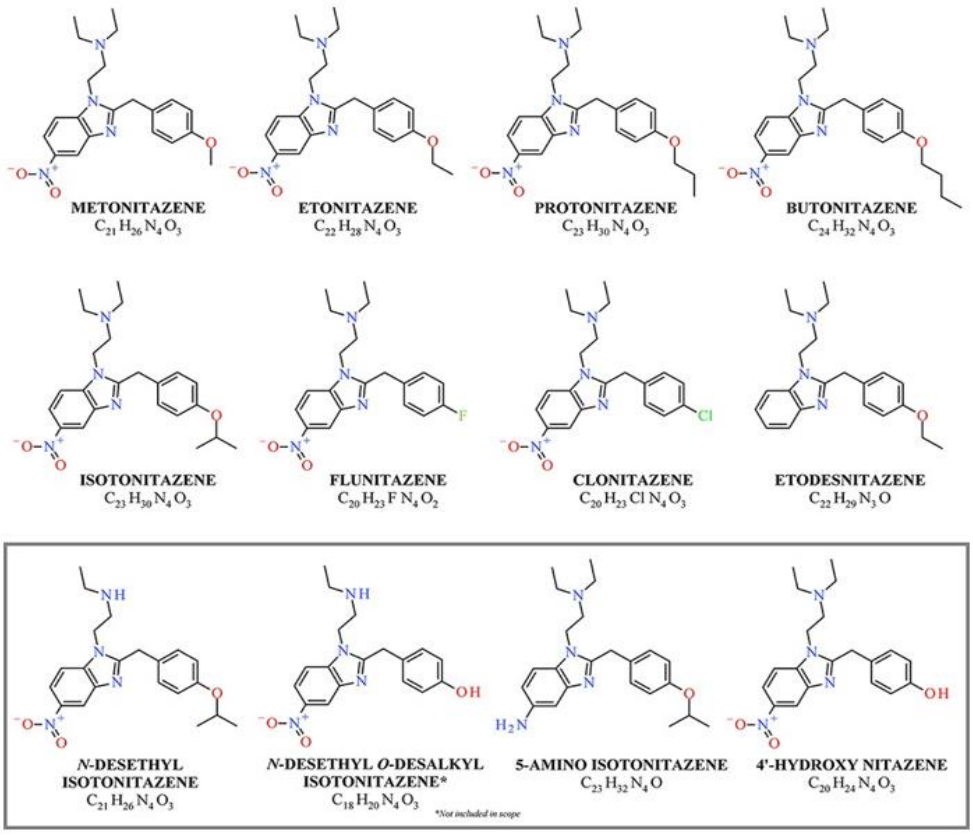


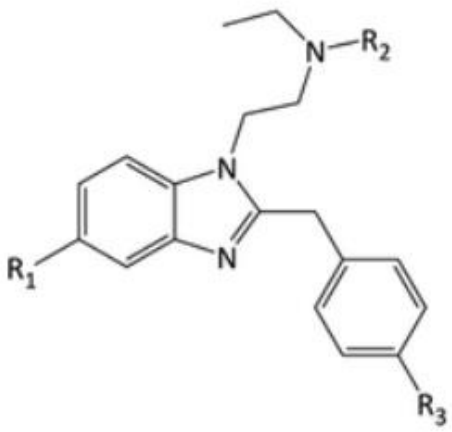
***Figure 2**. Structures of nitazene analogs included in analysis, as well as the metabolites of isotonitazene (box). Structures consist of the benzimidazole core, nitro group, alkoxy benzyl group and ethylamine component[11].*

A public alert released by the Center of Forensic Science Research and Education (CFSRE) was the first indication that the new nitazenes were entering the United States[12]. The CFSRE warned in November of 2019 that isotonitazene was making its way into Indiana and Illinois from Canada and Belgium, which had seen other overdose cases earlier the same year[12]. Between Indiana and Illinois there were eight postmortem blood samples that tested positive for isotonitazene[12]. This was the first time that isotonitazene was recognized as being as dangerous as etonitazene. The alert then goes on to give recommendations for public health departments and doctors in the case of a nitazene overdose as well as the demographics that are associated with nitazene use[12].

Also in November 2019, Peter Blanckaert et. al. published a paper delving into the detection of isotonitazene as a result of overdose cases from around the world. Blanckaert evaluated gas chromatography mass spectrometry (GCMS), liquid chromatography quadrupole-time of flight mass spectrometry (LC-QTOF-MS), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance spectroscopy (NMR) for the detection of isotonitazene[13]. The compound was further characterized it by studying the effects of isotonitazene in vitro on the μ-opioid receptor (MOR)[13]. As a result, researchers were able to determine a characteristic spectrum for each method as well as determine that isotonitazene has an extremely high MOR potency with an EC50 of 11.1 nM[13]. This paper provided inspiration for this project, as the goal was to characterize available nitazenes using GCMS and FTIR.

Soon after, in February 2020, Alex Krotulski et. al. published an article detailing 18 overdose cases in the United States where isotonitazene was found in the deceased's blood[14]. Each blood sample was analyzed for other opioids and the amount of isotonitazene was quantified[14]. This paper also identified and quantified several metabolites of isotonitazene in the blood samples using LC-QTOF-MS[14]. Also in 2020, the World Health Organization held a committee meeting on the increase of isotonitazene use among opioid users. This committee document covers may of the important facts regarding isotonitazene and how to anticipate and treat overdoses.

In 2021 there was further awareness about their potential danger of nitazenes to opioid users. In March 2021, Marthe Vandeputte et. al. expanded the characterization and synthesis of nitazenes to ten analogs and their metabolites.



	R_1	R_2	R_3
1. Isotonitazene	$-NO_2$	$-C_2H_5$	$-OCH(CH_3)_2$
2. *N*-desethyl-isotonitazene	$-NO_2$	-H	$-OCH(CH_3)_2$
3. 4'-OH-nitazene	$-NO_2$	$-C_2H_5$	-OH
4. 5-aminoisotonitazene	$-NH_2$	$-C_2H_5$	$-OCH(CH_3)_2$
5. Metonitazene	$-NO_2$	$-C_2H_5$	$-OCH_3$
6. Etonitazene	$-NO_2$	$-C_2H_5$	$-OC_2H_5$
7. *N*-desethyl-etonitazene	$-NO_2$	-H	$-OC_2H_5$
8. Protonitazene	$-NO_2$	$-C_2H_5$	$-OC_3H_7$
9. Butonitazene	$-NO_2$	$-C_2H_5$	$-OC_4H_9$
10. Clonitazene	$-NO_2$	$-C_2H_5$	-Cl
11. Flunitazene	$-NO_2$	$-C_2H_5$	-F
12. Isotodesnitazene	-H	$-C_2H_5$	$-OCH(CH_3)_2$
13. Metodesnitazene (metazene)	-H	$-C_2H_5$	$-OCH_3$
14. Etodesnitazene (etazene)	-H	$-C_2H_5$	$-OC_2H_5$

Figure 3 *The generic structure of nitazenes and a table listing the analogs and their substituents[6].*

Vandeputte et al. used LC-QTOF-MS, GCMS and high performance liquid chromatography diode array detection (HPLC DAD) for their detection and were able to determine the major ions, fragments and retention times for each nitazene compound[7]. This paper also detailed toxicological functions of nitazenes. They found that they are highly active at the MOR, exceeding fentanyl in their potencies[7]. Later the same year, Vandeputte et al. published yet another paper about isotonitazene and a similar synthetic opioid, brorphine[15]. This was a review of literature about isotonitazene and brorphine[15]. This paper stands out as it is the first to mention brorphine in conjunction with isotonitazene[15]. Though this project does not focus on brorphine, it is still important to note that there are other synthetic opioids similar to nitazenes that pose a threat to society.

An increase in fatalities due to isotonitazene overdose was noted in 2021. A paper published by F. Mueller et al. highlighted the first three reported deaths in Switzerland due to isotonitazene[16]. This study analyzed a homogenized sample of the first victim's lungs, liver,

kidneys, heart, brain, spleen, and femoral muscle for isotonitazene[16]. They used NMR, GC-FTIR, ATR-FTIR, and GCMS to analyze the sample and found that it had a 95-98% peak purity in the spectra (meaning that the peak showing up on the spectrum was isotonitazene with 95% certainty) and the concentration was 1.20 ng/mL[16]. Such a small amount of isotonitazene causing an overdose further established the extreme potency of isotonitazene. Another paper published in 2021 analyzed about 1000 overdose cases in Illinois and Wisconsin from January 2020 to July 2020[17]. They found that 40 of the overdoses in that 7 month period involved isotonitazene, often in tandem with benzodiazepines[17]. This study emphasizes that deaths involving isotonitazene often also involved significantly more substances compared to other synthetic opioid deaths[17]. The increase in polypharmacy in cases of nitazenes could indicate that users believe they are using a different drug, when in fact a nitazene (likely of a much higher potency) has been mixed in.

Later in 2021, a more in-depth study of another nitazenes analog metonitazene and its involvement in overdoses in the United States was done by Alex Krotulski et. al. from the CFSRE[18]. Their main methods of analysis were LC-QTOF for screening and LC-MS/MS for confirmation[18].They studied 20 postmortem cases where metonitazene was ruled to be the drug responsible for the cause of death, and every death was ruled to be an accident[18]. The average concentration of metonitazene found in the blood was 6.3 +/- 7.5 ng/mL and the average concentration in urine was 15 +/- 13 ng/mL and was the only drug identified in 30% of the cases[18]. In the other 70% of cases metonitazene was found in combination with fentanyl, benzodiazepines, opioids, and hallucinogens, enforcing the hypothesis that users do not know they are getting nitazenes in their drugs[18].

Another paper that heavily influenced this project was a review done by Istvan Ujvary et al. that details all the characteristics of 2-benzylbenzimidazole opioids to date[19]. This paper covers all of the manufacturing information, toxicological information, pharmacology, and addiction potential of nitazenes[19]. Ujvary provides an excerpt from the original Clinical Investigation Report explaining the reasoning behind stopping the clinical trials when nitazenes were initially discovered in the 1950s[19]. It shows that the main reasons for the discontinuation were that nitazenes had no distinct advantages over morphine and its analgesic effect was weak and irregular when administered orally[19].

As the project progressed, etonitazepyne became an increasingly interesting analog. In June 2021, Peter Blanckaert et al. published a paper characterizing etonitazepyne, a colloquial name for N-pyrrolidino etonitazene [20].

Figure 4 *A bag of etonitazepyne that Blanckaert et al. purchased for their study[20].*

They analyzed etonitazepyne using GC-MS, LC- MS/MS, NMR and FTIR. Their results for GC-MS had very similar major ions as the other main analogs as well as similar retention times.

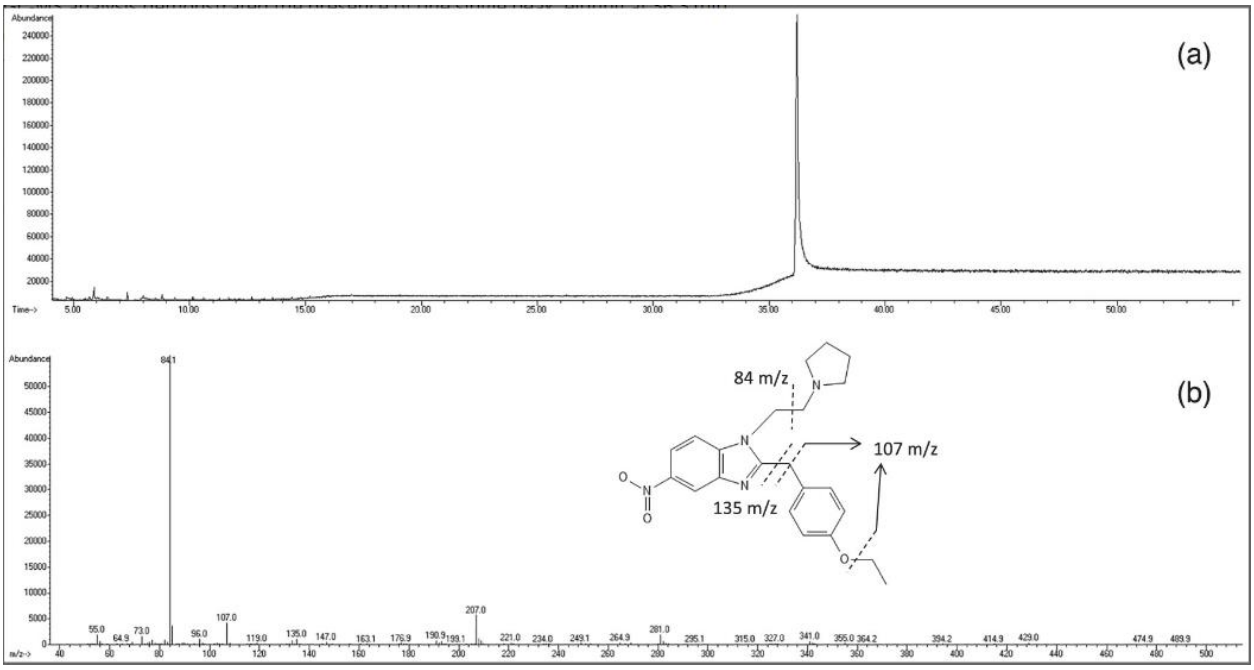


Figure 5 *Total ion chromatogram and mass spectrum of etonitazepyne[20].*

Blanckaert goes on to say that according to online Reddit forums, the compound is clearly an active opioid, as users were interested in a substance that was potent, but not scheduled[20]. Later, in 2022, Vandeputte et al. published a paper with the National Institute of Justice expanding on the character of etonitazepyne by studying its pharmacological effects[21]. They determined that etonitazepyne also has a high affinity for the MOR with 21 overdose fatalities associated with it, two of which where it was the sole cause of death[21].

Beginning in 2022 scientists were trying to design methods to combat synthetic opioid overdoses. In January 2022, Jinny Claire Lee et al. developed an immunopharmacotherapy to treat cases of synthetic opioid overdoses[22]. Antibodies were developed which bound to the opioids themselves so that they could not bind to the opioid receptors in the human body[22]. The

antibodies would reduce the effect that NPS would have on the brain, and lessen the risk of an overdose[22]. They were also able to provide further insight into the unreported pharmacokinetics of nitazenes[22].

Meanwhile, Adam White et al. proved that naloxone, a medication that binds to opioid receptors to reverse an opioid overdose, can reverse a metonitazene overdose[23]. In the case that White et al. observed, only one dose of naloxone was needed to revive the user, however they acknowledge that with users that had taken a higher dose of metonitazene, then a larger dose of naloxone would be needed[23]. A third paper written by Phil Skolnick in 2022 reviewed the current methods to combat overdoses, including naloxone and naloxone alternatives[5]. Skolnick lists some naloxone alternatives such as AMPA receptor potentiators, serotonin receptor agonists, calcium activated potassium channel blockers and cyclodextrin scaffolds that would bind to the nitazene itself[5]. He also mentions that MOR antagonists with a higher affinity than naloxone have been developed and approved by the FDA[5]. However, he does acknowledge the drawbacks to using MOR antagonists, the most prominent being that there is potential for severe withdrawal in users who are opioid dependent and could potentially cause heart problems[5]. Skolnick's response to this is that the symptoms of withdrawal are not life-threatening and it is imperative that new approaches to opioid overdoses be a public health priority[5]. A study done on naloxone use later in 2023 concurred with these findings. Alexandra Amaducci et al. studied overdose patients in emergency departments who tested positive for synthetic opioids and found that metonitazene overdose patients overall a higher dosage of naloxone to reverse clinical signs- these patients were also more prone to cardiac arrest[24].

The analog protonitazene began to be recognized as dangerous in 2022. The World Health Organization (WHO) put out a report from their Expert Committee on Drug Dependence

reviewing all of the characteristics of protonitazene[25]. The report details information on the MOR studies, as well as toxicological studies[25]. The WHO reports that protonitazene has no therapeutic applications and that it has been detected in Canada, the United States and Australia in overdose cases[25].

At the end of 2022, a summary of drug spectra was published using the GCMS data collected for many categories of drugs[26]. In the section about nitazenes, William Feeney et al. described the mass spectra as having several noteworthy ions for identification, including m/z 86, m/z 107, m/z 207, and m/z 235[26]. They determined that characteristic ions are the 86 and 107 ions and that there will be few ions above 120 with common neutral losses of 261 Da and 310 Da[26].

In 2023 interest in nitazenes increased, use became more common globally. The development of rapid screening tests was developed to help combat the rising tide. Martin Kimani et al. used FTIR coupled with direct analysis real time ambient ionization coupled to a thermal desorption unit and a mass spectrometer, Raman spectroscopy, surface enhanced Raman scattering for rapid screening, and LC-MS to confirm their presence[27]. These methods were able to screen suspect tablets for opioids and were able to identify nitazenes. However they were not able to determine the specific analog, due to structural similarities[27]. Using LC-MS, the tablets were found to have an average of 0.817 mg of etonitazepyne per tablet, confirming the positive results of the preliminary methods employed[27]. Another handheld rapid testing unit making use of Raman spectroscopy, TruNarc (Thermofisher, Waltham, Massachusetts), began being used by police officers to identify nitazenes in the field[28]. The library of the TruNarc has been updated to include several new nitazenes among a host of other novel drugs and allowing officers to decide quickly what protocols to follow[28]. The DEA also decided that the nitazenes had gone

unscheduled long enough and scheduled metonitazene on August 18, 2023, and n-desethyl isotonitazene and n-piperidinyl etonitazene on October 25, 2023 into Schedule 1[29,30].

Countries all over the world have been reporting cases of nitazene overdose. Japan reported a postmortem case in which the victim had 6.0 ng/mL of metonitazene in their blood and 5.2 ng/mL of metonitazene in their urine[31]. Several cases of overdoses also emerged in the United Kingdom. A CFSRE warning mentioned that the United Kingdom was seeing overdose cases where the blood concentrations for n-pyrrolidino protonitazene ranged from 0.1 to 55 ng/mL[32]. Another article from the BBC warned Scottish citizens that 54 deaths had been linked to nitazenes over a six month period in 2023[33]. A paper published at the end of 2023 discussed the decline of heroin use in Europe and the rise of synthetic opioid use[34]. Nitazenes are responsible for a rapid escalation of opioid related deaths since 2022 and European officials found them mixed with benzodiazepines and xylazine[34]. In Australia, synthetic opioids were being purchased with cryptocurrency, a terrifying combination of technology and drugs[35]. The nitazenes that appeared in these transactions were likely used as adulterants for other drugs such as cocaine[35]. Other North American countries did not escape the flood of synthetic opioids, as in July 2023, Canada reported that overdoses due to these drugs were on a steady rise[36]. R. Michael Krausz et al. propose possible solutions to combat drug abuse including more treatment centers for those addicted as well as creating a "safe supply" of opioids so drug users are not exposed to worse substances used to cut them with[36]. In October 2023, DEA Administrator Milgram delivered a speech about nitazenes, along with other synthetic opioids, being made in China and sold in the United States through cartels[37]. Drugs were being sold online for pennies or cryptocurrency and sent through the postal service, packaged in such a way to circumvent

customs investigators[37]. This led to yet another wave of opioid addiction in the United States, as evidenced in several specific cases.

In New York City, 14 overdose cases led to investigators developing a method to quantify metonitazene and isotonitazene in plasma, blood, urine, liver, and brain tissues[38]. Using LC-MS, they found metonitazene in concentrations of 0.10 to 1.5 ng/mL in four postmortem analyses and isotonitazene in concentrations of 0.11 to 0.12 ng/mL in ten postmortem analyses of femoral blood[38]. They judged all the deaths in these cases were due to accidental overdoses and in none of the cases were nitazenes the sole intoxicant responsible for death[38]. Wastewater in Arizona, Oregon, New Mexico, Illinois, New Jersey, Washington, and Georgia was collected and analyzed for nitazene analogs[39]. Richard Bade et al. found protonitazene in wastewater in Washington and Illinois with concentrations of 0.5 ng/L and 0.2 ng/L respectively using LC-MS[39]. This study is the first of its kind and was able to develop a method for detecting nitazenes in wastewater. In order to achieve usable results, they pre-concentrated their samples and used extremely sensitive instrumentation to detect such low concentrations[39]. Such amounts in wastewater indicates just how large of a problem nitazenes are becoming. Nitazene detection and an overdose in Colorado exemplify the western movement of nitazenes use across the country. In the last few months of 2023, Boulder, Colorado had an overdose due to n-desethyl etonitazene, the first death due to nitazenes in the state[40].

Both the National Institute of Justice and the Alcohol and Drug Foundation have recognized the need to spread the word about the new nitazenes entering the country and have published articles explaining the forms that nitazenes come in and what their effects are for short term use and long term withdrawal effects[41,42]. They warn folks of polypharmacy and its dangers

as well as try to lessen the harm that these drugs are doing to addicts by teaching them about what they are using[41,42].

In June 2023 there were two other articles about nitazenes of note. The first is a paper by Grant Glatfelter et al. describing how the alkoxy chain length governs the potency of the 2-benzylbenzimidazole species[43]. They found that etonitazene, with an ethoxy chain, was the most potent out of all of the nitazene analogues that they tested and that overall ethoxy, isopropoxy, and propoxy chains were attached to analogues with higher potencies than fentanyl and that methoxy and butoxy chains led to lower potency[43]. They discovered this by performing MOR functional assays in mice and in vivo severe acute respiratory syndrome (SARS) tests[43]. The other article to come out was another review of nitazene literature which discussed their entry into the illicit drug supply, clinical implications, and public health implications[44]. They concluded that nitazenes are likely to continue to stay in the drug market for a long time and that more analogs will be developed as the existing ones get scheduled[44]. They also warned that synthetic opioids of this type are not difficult for chemists to make and that there will be increasingly deadlier substances that will be introduced to the street drug market[44]. The healthcare system needs to be better educated about nitazenes and better equipped to handle the rising number of overdoses by learning better treatment methods and having more efficient detection systems[44].

As this thesis is being prepared, few articles about nitazenes have appeared in 2024. The DEA put out a fact sheet explaining the dangers of nitazenes and giving a short summary of their chemistry, pharmacology, user population, illicit distribution, and control status[45]. Forbes also featured a warning about nitazenes in their innovation section of their website and designated it breaking news[46]. The article gives key information about nitazenes that is distributed to the wider

audience of Forbes to help them realize the threat[46]. Europe is experiencing continuing nitazene overdoses in 2024 as noted by a Norwegian article published in the Journal of the Norwegian Medical Association[47]. A young man overdosed on an opioid nasal spray that he did not realize was spiked with protonitazene[47]. Initially the blood work came back negative for routine drugs, so hospital workers had to carry out a "broad substance search" to identify protonitazene in the patient's blood[47]. This is yet another story of regular drug users being unaware of the drugs they are taking and then overdosing on nitazenes because they weren't prepared. The United Kingdom is also becoming increasingly worried about their drug crisis[48]. They acknowledged that nitazenes are heralding a second wave of drug related deaths and emphasized that public health officials should be on the lookout for these potent synthetic opioids[48].

Meng-Hua Tsai et al. published a paper this year showing that nitazenes have a greater potency and efficacy in the cAMP and β-arrestin2 assays than fentanyl and its analogs[49]. This means that 2-benzylbenzimidazole analogs have a higher efficacy when activating the Gi protein, causing a higher risk for intoxication and overdose[49]. Lastly, in 2024, a paper that is going to be published in the April edition of the Journal of Pharmaceutical and Biomedical Analysis explains an LC-MS method to quantify nine nitazene analogs in dried blood spots[50]. They extracted the analogs into methanol: acetonitrile (3:1), dried it down, reconstituted in 30 µL of methanol and injected it onto the instrument with a fentanyl D5 internal standard[50]. They were able to detect concentrations as low as 1 ng/mL of each analog, but mentioned that future research could be done on mixtures of drugs containing nitazenes to ensure that the method could work for all situations[50].

Although this project is not novel in the field of forensic science, it is important, nonetheless. Nitazenes are moving across the United States and labs everywhere will need to

prepare for the onslaught of nitazene analogs that are taking over the opioid drug scene. More presumptive tests unrelated to QTOF need to be developed for nitazenes if we are to keep lab work quick and inexpensive. This project will ensure that Sacramento County District Attorney's Laboratory of Forensic Services will have a working method in their Controlled Substances section that can detect nitazenes and identify counterfeit pills.

Chapter 2: Materials and Methods

Standards

All standards were purchased from Cayman Chemicals (Ann Arbor, Michigan). Their lot numbers are: 0641452-15 for nitazene citrate, 0586760-42 for isotonitazene, 0608348-40 for metonitazene, 0590271-26 for protonitazene, 0589128-46 for etonitazene and 0607145-16 for etonitazepyne. In addition to the standards directly purchased from Cayman, several counterfeit pills were pulled from casework and examined. There were three colorful pills from one case and a fake oxycodone pill from a different case. See pictures in the results and discussion section.

Color Tests

Color tests were performed on nitazene citrate, metonitazene, isotonitazene, and etonitazepyne. About 10 mg of each standard was used for each set of color tests. For nitazene citrate, metonitazene, and isotonitazene each color test was done in triplicate to ensure the reaction was consistent. They could not be performed on protonitazene and etonitazene because there was not enough of the standard purchased. The color tests performed were Marquis, nitroprusside, cobalt thiocyanate, Mecke, Froehde, Van Urk, Wagner, Dille- Koppanyi, Liebermann's and ferric chloride. The Marquis reagents were formaldehyde from EMD (Damstadt, Germany) and concentrated hydrochloric acid from JT Baker (Phillipsburg, New Jersey). The nitroprusside reagents were sodium nitroferricyanide from JT Baker and acetaldehyde from Sigma Aldrich (St. Louis, Missouri) for reagent A, and sodium carbonate from Sigma Aldrich in water for reagent B. The cobalt thiocyanate was from Sigma Aldrich and the stannous chloride is from Spectrum Chemical (New Brunswick, New Jersey). Mecke reagent A was selenious acid from Sigma Aldrich and reagent B was sulfuric acid from JT Baker.

Froehde reagent A was molybdic acid from JT Baker and reagent B was sulfuric acid also from JT Baker. Van Urk reagent A was paramethylaminobenzaldehyde from Sigma Aldrich and reagent B was methanol from VWR International (Radnor, Pennsylvania). Wagner reagent A was hydrochloric acid from JT Baker, and reagent B was a mixture of iodine and potassium iodide, both from Sigma Aldrich. Dille Koppanyi reagent A was a mixture of cobalt acetate from Matheson, Coleman and Bell (Norwood, Ohio, Los Angeles, California, and East Rutherford, New Jersey), methanol from VWR, and glacial acetic acid from JT Baker. Dille Koppanyi reagent B was a mixture of isopropyl amine from Sigma Aldrich and methanol from VWR. Liebermann's reagent was a mixture of potassium nitrite from Sigma Aldrich and sulfuric acid from JT Baker. The ferric chloride test was done with ferric chloride from EMD.

Extractions

Three extractions were performed on metonitazene, nitazene citrate, and isotonitazene to determine which would yield the best results on the GCMS. For each extraction, 1.2 mg of the standard was weighed out on an Ohaus (Parsippany, New Jersey) Discovery balance. The metonitazene standard was a liquid, so 0.5 mL (equivalent to 0.5 mg) were used instead. The first extraction tried was a basic extraction using 0.5 mL of NaOH from Sigma Aldrich, one drop of methapyrilene from Sigma Aldrich as an internal standard and 0.5 mL of toluene from Sigma Aldrich. The chemicals were all pipetted using an ASA Proline Plus 100-1000µL pipet from Thermofisher (Waltham, Massachusetts). This mixture was vortexed and then, using a disposable pipet, the top layer was added to a GCMS vial in an insert. The second extraction tried was a pH 9 extraction mixing the standard with 0.5 mL of Borax pH 9 buffer (made up of sodium borate decahydrate from JT Baker), one drop of methapyrilene internal standard, and 0.5 mL 3:1 mixture of chloroform from JT Baker and isopropyl alcohol from Sigma Aldrich. This

mixture was vortexed, and the bottom layer pipetted using a disposable pipet to a GCMS vial insert. The last extraction method attempted was a simple methanol dissolution, where the standards were dissolved in 0.5 mL of methanol from VWR, and one drop of external standard. The external standard was still methapyrilene, but in toluene instead of deionized water. Blanks were run for each sample as well, with just the extraction reagents.

Gas Chromatography- Mass Spectrometry

The nitazene standards were run on two different GCMS instruments. The first (Instrument 11) had an Agilent Technologies (Santa Clara, California) 7890B GC System coupled to a 5977A Mass Selective Detector (MSD) with a 7693 Autosampler. The second (Instrument 9) had an Agilent Technologies 6890N Network GC System coupled to a 5973 Inert MSD with a 7683 series autosampler. The column used on both instruments was an Agilent Technologies 122-5512 DB-5MS column with a length of 15 meters, 0.25 mm diameter, a 0.25 µm thick film with temperature parameters of -60-325°C, and serial number US2530625.

Table 1 *Parameters for GC-MS methods.*

	Temperature Range (°C)	Ramp Rate (°C/min)	Split Ratio	Flow Rate (mL/min)	Length (min)	Injection Volume (µL)
Drug	90-300	60	20:1	1.0	10	0.2
Drug 2x	90-300	60	10:1	1.0	10	0.2
Steroid	90-280	20	20:1	1.0	25	0.2
Nitazene	90-280	20	30:1	1.0	15	1.0

There were four methods run on the standards during the course of the project: drug, drug 2x, steroid, and nitazene. All methods used helium as the carrier gas. The fourth method was the one created in this project. Starting with the steroid method as a base, the nitazenes method ended up with the same temperature ramp rate and range as the steroid method but the final hold was only 9 minutes. The method length was changed to be 15 minutes instead of 25 minutes and the split ratio changed to 30:1 instead of 20:1. The largest change made was adjusting the injection volume from 0.2 µL to 1.0 µL. Though it seems counterintuitive to increase the split and the injection volume, there was no option to change the injection volume to 0.5 µL. There was a concern with overloading the instrument, and as a result the split was increased. Each standard was run on this method and produced comparable results to the premade methods that the lab already had. After each standard had been run, they were added to the instruments' internal drug libraries using the Mass Hunter analysis program.

Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance

FTIR-ATR was run on nitazene citrate, isotonitazene, protonitazene and each was added to the instrument's internal drug library. Metonitazene and etonitazepyne were not added because they were in liquid solutions when initially received and only the solvent would show up in the IR spectrum. Etonitazene was not added because there was not enough standard to put onto the sensor of the IR. About 1 mg of each standard was put onto the crystal of the ATR attachment and run using FTIR. The infrared spectrometer used was a Thermo Scientific Nicolet iS10 #2, with serial number AKX1100932, and ATR attachment X82224, 869-174400 iTX Base. For each analysis 64 scans of the background and of the substance being analyzed were done to mimic the number of scans typical for casework. It is worth noting that only pure standards and

drugs can be run on IR, so this technique is only advised when the analyst knows the purity of the substance they are analyzing.

TruNarc

The standards were also analyzed by a new presumptive test that prisons are using to identify drugs, the TruNarc. It is a handheld Raman spectrometer developed by Thermofisher designed for police officers on patrol to quickly test drugs through their packaging, reducing the risk of the officer coming into contact with the drug itself. There is no preparation needed for the drug and it can be kept in its original packaging. To prep the instrument, a check must be run on the polystyrene standard that comes attached to it and then it is ready for use, assuming the test comes back indicating that the instrument is functioning. The "unknown" drug standard is then placed in front of the laser and scanned for about 1-2 minutes. The screen will then display whether or not the substance is an illicit drug and identify the substance. It is important to make sure that the laser is directly on the substance that is being analyzed otherwise there will be an inconclusive result. Initially when this instrument was tried, there was an outdated library so none of the nitazene standards came back as conclusive. However, since then the library has been updated to v1.10 and the TruNarc is able to get a conclusive reading on isotonitazene. The others were inconclusive because they were either a liquid, or there was not enough of the standard for the TruNarc to get a good reading. The lab has two TruNarcs with TN#1 having the serial number 222627 and TN#2 having the serial number 222694.

Chapter 3: Results for Isotonitazene, Metonitazene, Nitazene Citrate

Color Tests

The first three analogs characterized were isotonitazene, metonitazene, and nitazene citrate. When ordering the standards, 50 mg of isotonitazene and nitazene citrate and 5 mg of metonitazene in acetonitrile were received. First, color tests were performed on the nitazene standards. Color tests were also performed on the commonly abused drugs that they were meant to react with so that there would be a positive control for comparison.

Figure 6 *Positive controls for known drugs: 1= heroin,2= codeine,3=hydrocodone, 4=methamphetamine,5=amphetamine, 6= cocaine HCl, 7=cocaine base, 8= diazepam, 9= psilocybin, 10= pentobarbital, 11= GHB, 12= caffeine.*

In addition to the nine tests that the lab normally does, Liebermann's test for alkaloids was added. Alkaloids will turn yellow, orange, or brown when this test is positive. Liebermann's is especially important for nitazenes, as the base structure, as seen in Figure 3, has several nitrogen containing groups and it is expected that they will all have a color change when this reagent is added. Liebermann's reagent was also applied to the same drug standards in Figure 6 to

determine the color change that would occur for those drugs and if it would be similar to the nitazenes.

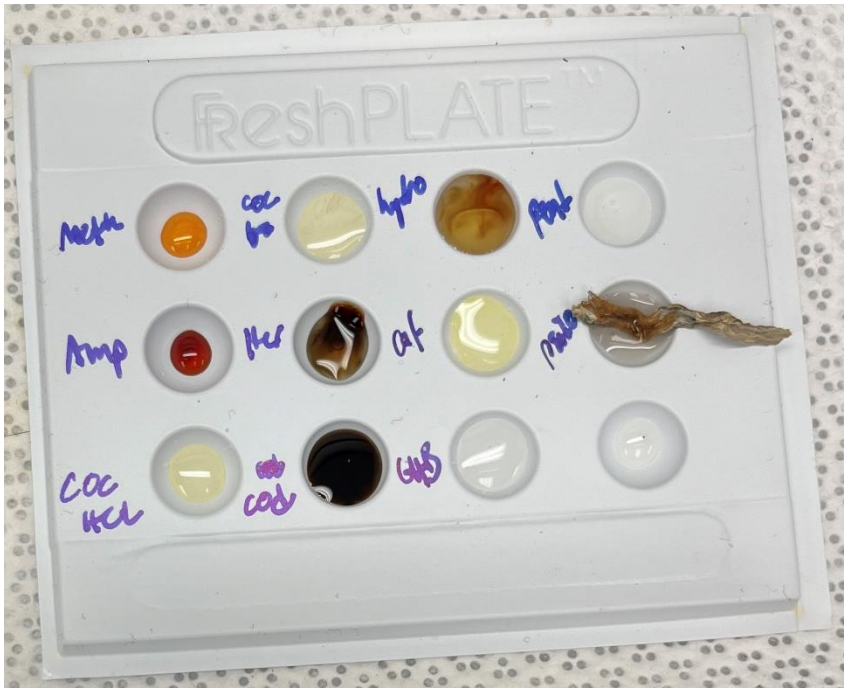


Figure 7 *Liebermann's color test (moving top to bottom and left to right) methamphetamine, amphetamine, cocaine HCl, cocaine base, heroin, codeine, hydrocodone, caffeine, GHB, pentobarbital, and psilocybin.*

Figure 7 shows that the only drug standards that did not have a color change indicating alkaloids were psilocybin, GHB, and pentobarbital. Though psilocybin and pentobarbital have nitrogen groups, it does not react with them because their nitrogens are acidic, and the test reacts only with basic nitrogens. GHB does not contain nitrogen, as such, did not react. Nitazene citrate had a color change with the nitroprusside test, the cobalt thiocyanate test, the Wagner test, and the Liebermann's test. Isotonitazene had a color change with the Marquis test, nitroprusside test, the cobalt thiocyanate test, the ferric chloride test, the Mecke test, the Froehde test, the Wagner test, and the Liebermann's test. Metonitazene had a color change with the Marquis test, the

nitroprusside test, the cobalt thiocyanate test, the ferric chloride test, the Mecke Test, the Froehde test, the Wagner test, and the Liebermann's test.

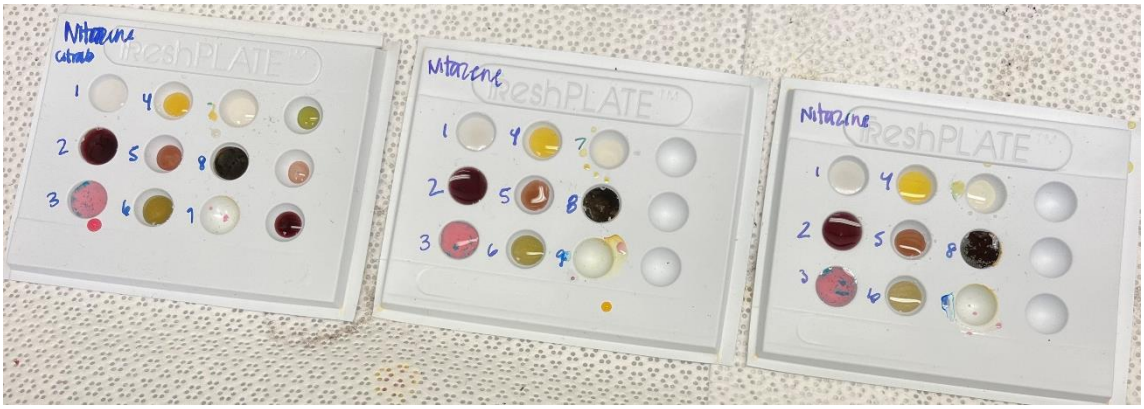


Figure 8 *Nitazene citrate color tests. 1. Marquis 2. Nitroprusside 3. Cobalt thiocyanate 4. Ferric chloride 5. Mecke 6. Froehde 7. Van Urk 8. Wagner 9. Dille Koppanyi*

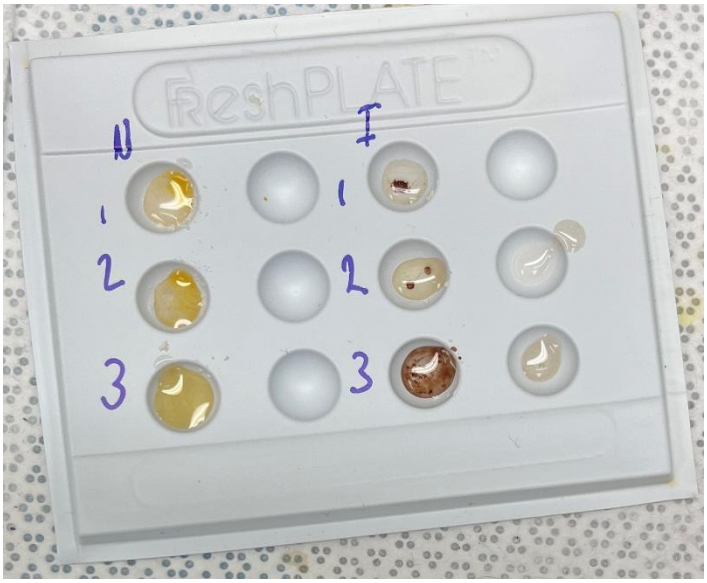


Figure 9 *Liebermann's test for nitazene citrate and isotonitazene. N is nitazene citrate and I is isotonitazene.*

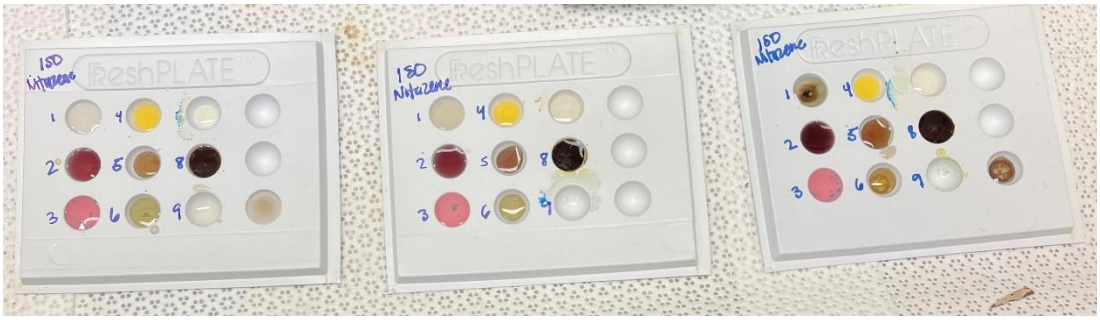


Figure 10 *Isotonitazene color tests. The numbers represent the same tests as in* ***Figure 8.***

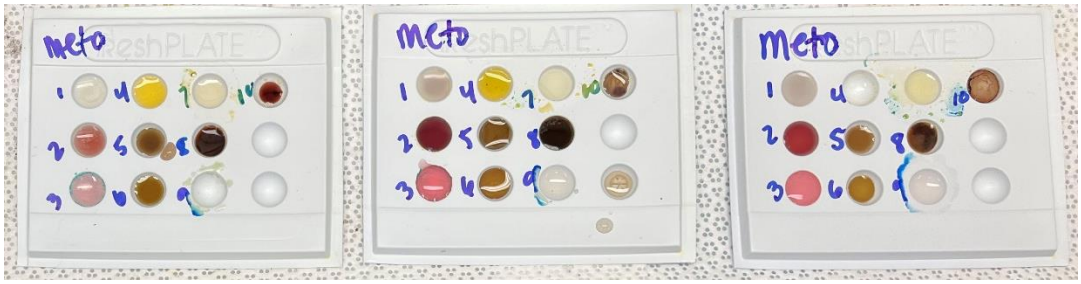


Figure 11 *Metonitazene color tests. The numbers represent the same tests as in* ***Figure 8****, with the addition of 10 being Liebermann's test.*

Table 2 *Color test results for the nitazene standards.*

	Marquis	**Nitro-prusside**	**Co(SCN)2/ SnCl2**	**Ferric Chloride**	**Mecke**	**Froehde**	**Van Urk**	**Wagner**	**Dille-Koppanyi**	**Liebermann's**
Steps	1. H2SO4 2. Formald.	1.Nitroprusside 2. Saturated Sod.Carbonate	1. Cobalt Thiocyanate 2. Stannous Chloride	1. Ferric Chloride 2. DI water	1. Selenious acid 2. Sulfuric Acid	1. Molybdic acid 2. Sulfuric Acid	1. Van Urk reagent 2. Con. HCl	1. 0.1N HCl 2. Wagner reagent	1. Rgt A 2. Rgt B	1. Potassium nitrite 2. Sulfuric acid
Compound										
Nitazene Citrate	No reaction	Faint red	Blue specks	No reaction	Tan precip.	No reaction	No reaction	Shiny brown precip	No reaction	Orange
Isotonitazene	Brown	Faint red	Blue specks	Orange specks	Orangey brown	Blue/green	No reaction	Shiny brown precip	No reaction	Brown
Metonitazene	Brown	Faint red	Blue specks	Orange specks	Brown	Brown	No reaction	Shiny brown precip	No reaction	Brown
Etonitazepyne	Brown	No reaction	No reaction	No reaction	No reaction	Brown	No reaction	Shiny brown precip	No reaction	Bubbles, gray/green

None of the analogs reacted with Van Urk and Dille Koppanyi because those tests do not look for alkaloids, but for specific drugs. Van Urk is sensitive to LSD and Dille Koppanyi is sensitive to barbiturates.

Extraction Method

After the color tests, the best extraction method was chosen for each analog. Of the three extraction methods tried, base extraction, pH 9 extraction, and methanol dissolution, the best was base extraction. Base extraction was deemed the best because it produced the sharpest peaks with the highest abundance. Each extraction technique was tested on all three analogs multiple times. The addition of the internal standard is to ensure that the instrument is performing as it should, this is also the reason for the blanks. The internal standard, methapyrilene, goes through all of the extraction steps that the drugs do so that if any one step goes incorrectly, it is immediately obvious. This is due to the fact that opioids need to be in the neutral form to be injected onto the GCMS. Though all of the extraction methods accomplished the goal of extracting the nitazenes, base extraction ensured that the analogs would not be ions. The basification ensures that a hydrogen does not bond to the nitrogens and create a positive charge. If a portion of the nitazenes have a positive charge, they will not extract into the toluene and the abundance of the peaks would be much lower. The methanol dissolution was sufficient to extract the nitazenes, however it was not very discerning about what else would be extracted and unwanted compounds diluted the standard, reducing the abundance of the peak. The pH 9 extraction was sufficient in terms of extraction, but because the pKas of isotonitazene and metonitazene are 8.22 and 9.91 respectively, and the pH extraction means not all of the molecules are neutral and lowers the abundance[51,52].

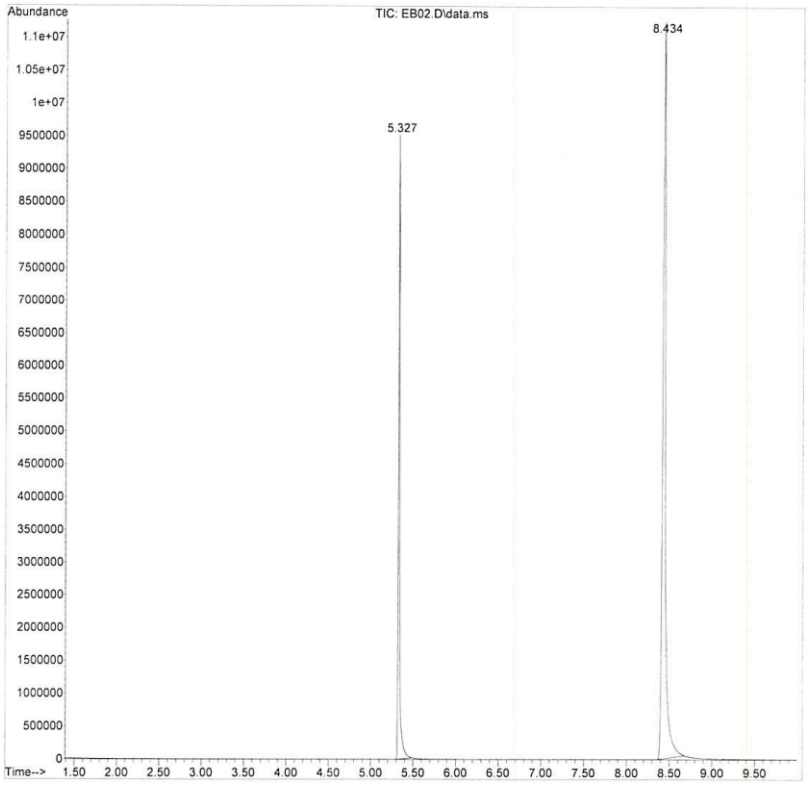


Figure 12 *Chromatogram of nitazene citrate (8.434 min) using base extraction.*

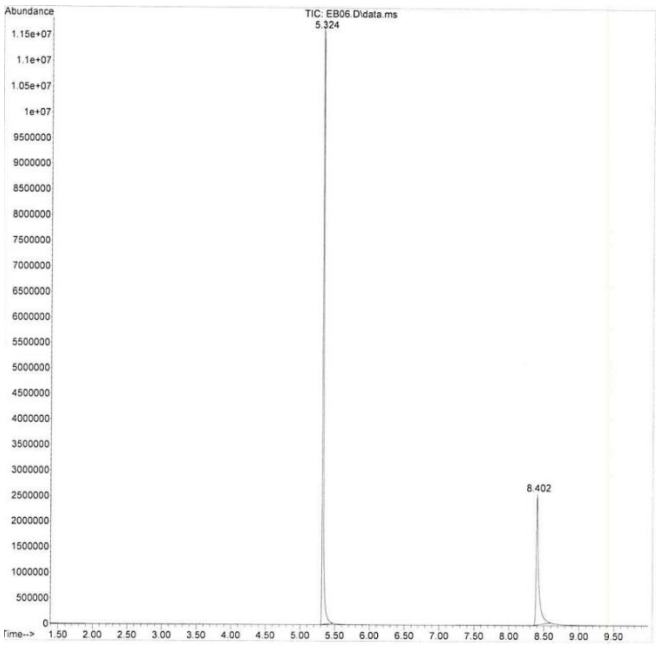


Figure 13 *Chromatogram of nitazene citrate (8.402 min) using pH 9 extraction on the Drug2X method.*

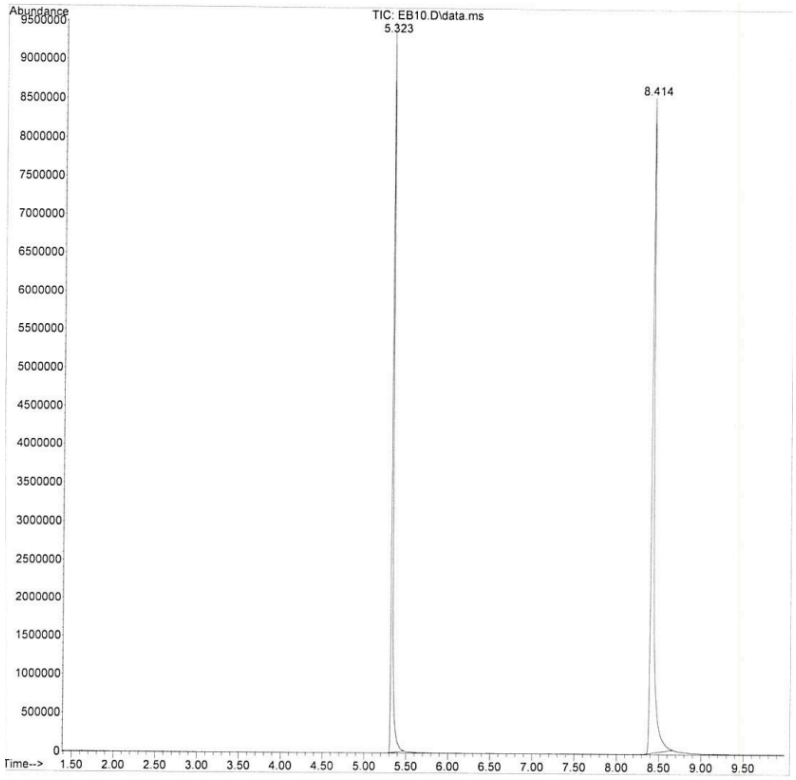


Figure 14 *Chromatogram of nitazene citrate (8.414 min) using methanol dissolution.*

The abundances in figures 13 and 14 are clearly much lower than that in Figure 12, so base extraction was deemed the best extraction method. The same can be said about isotonitazene and metonitazene and those chromatograms can be found in the appendix of this document.

Each nitazene analog had a retention time of around 10 minutes. Nitazene citrate has a shorter retention time than the other two because it is a smaller molecule and is less polar without the extra oxygen molecule the others have. Both isotonitazene and metonitazene had to be run on the lab's steroid method because the drug method only runs for 10 minutes and their retention times are 11.005 min and 10.139 min respectively, just outside of that time frame. When designing a method later, this was taken into consideration, as it is unwise to waste time running a 25-minute method like the steroid method, when a 15-minute method would perform the same purpose.

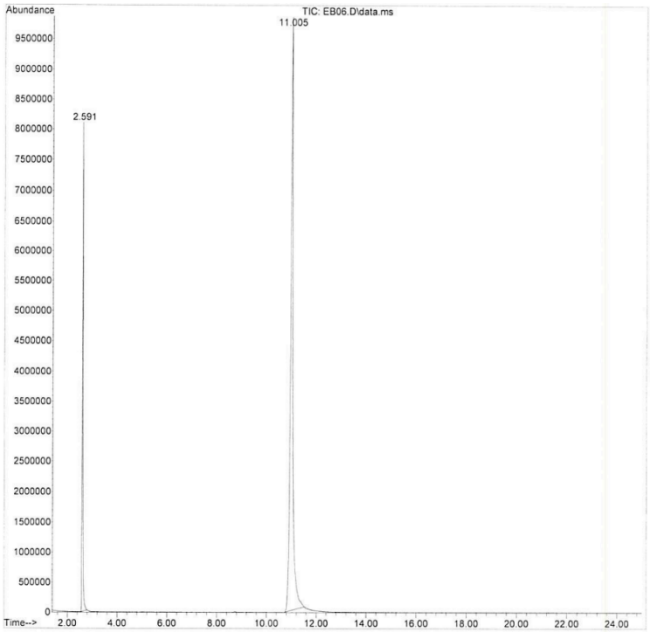


Figure 15 *Chromatogram of isotonitazene using base extraction and the steroid method.*

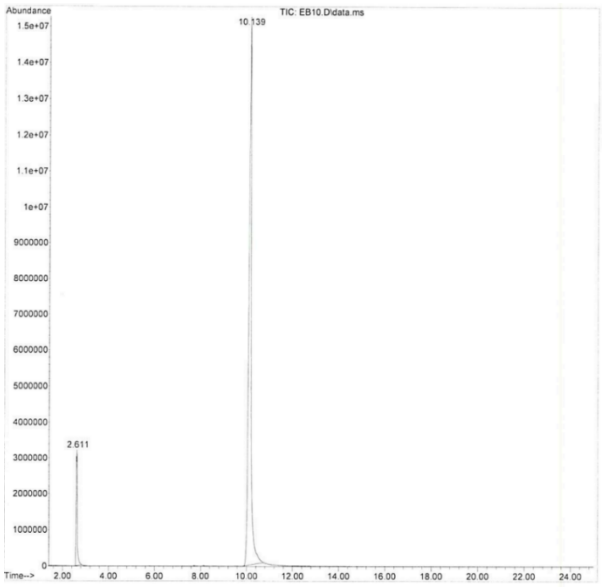


Figure 16 *Chromatogram of metonitazene using base extraction and the steroid method.*

Fragmentation

In addition to having similar retention times, these three analogs also had similar fragmentation patterns given their same base structure. The base peak for all three analogs is at 86 m/z. Though all of the compounds have interesting unique ions, a library match was used to ultimately decide which was which analog. The unique ions were used to aid in identification. Nitazene citrate's next most prevalent ion is at 90 m/z. Isotonitazene's ion is at 107 m/z. Metonitazene's ion is at 121 m/z.

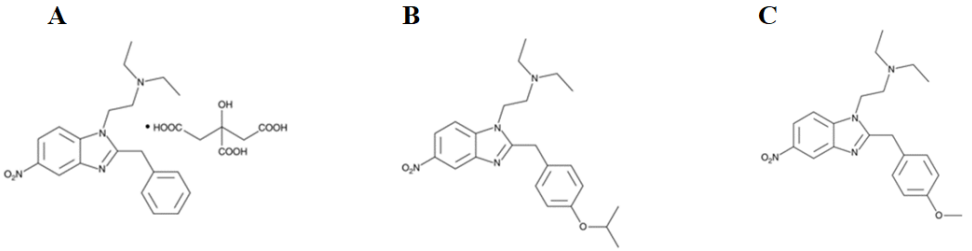


Figure 17 *Structures of the three analogs characterized here. A) nitazene citrate B) isotonitazene C) metonitazene*

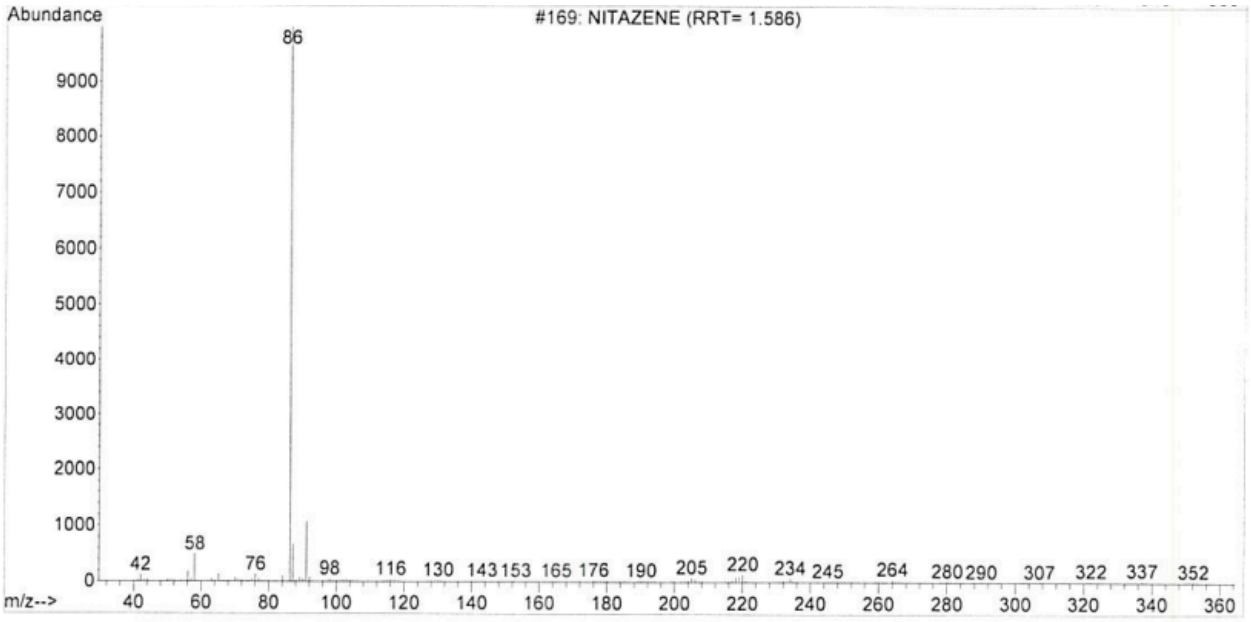


Figure 18 *Mass spectrum of nitazene citrate. The major ion at 90 m/z is not labelled but significant, nonetheless.*

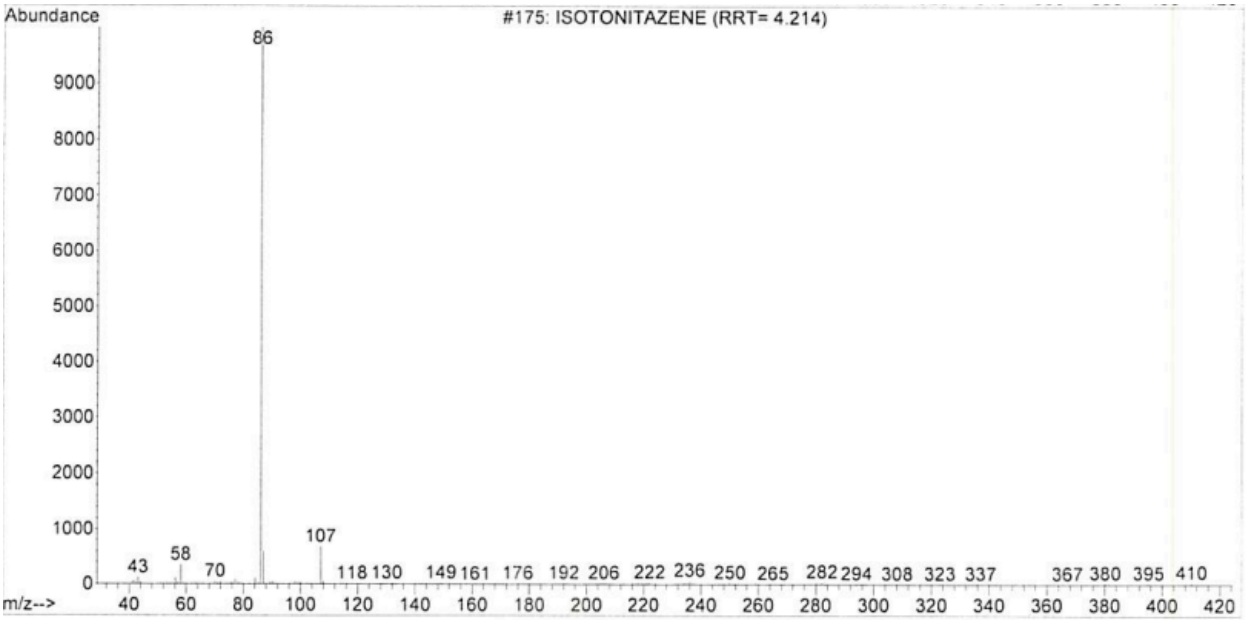


Figure 19 *Mass spectrum of isotonitazene.*

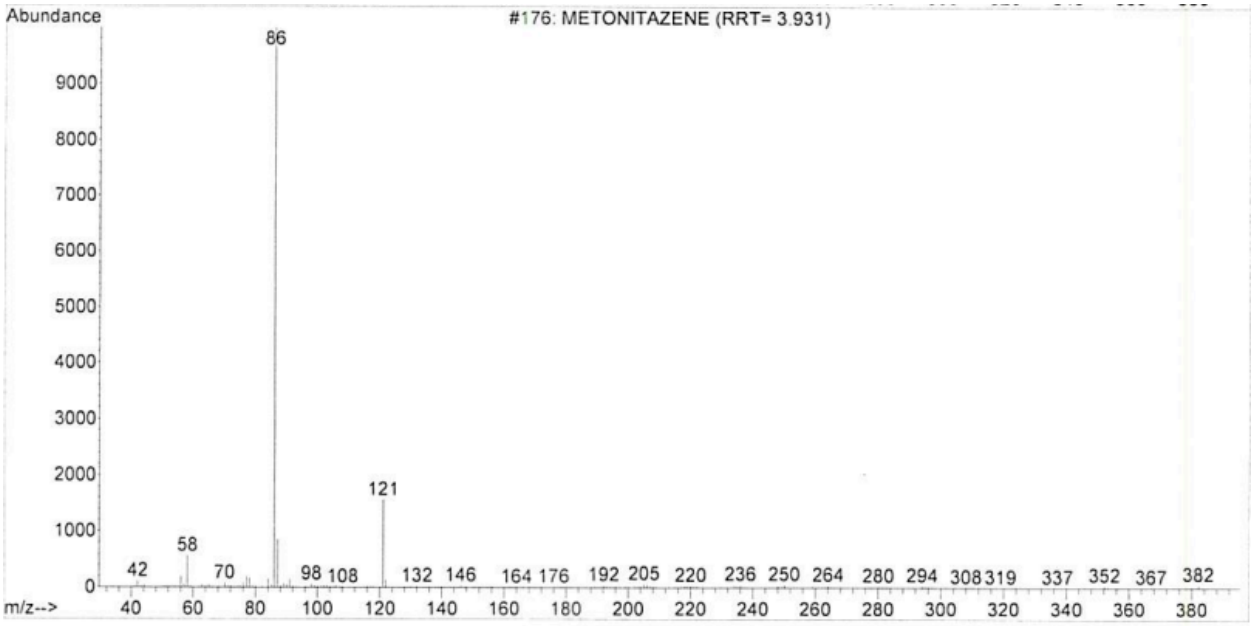


Figure 20 *Mass spectrum of metonitazene.*

Method Development

The steroid method that the lab uses was too long and was not as efficient as the method developed here. The next step was to develop a method similar to the Drug 2X method for the nitazenes. For GC-MS optimization, Instrument 9 was chosen given its availability, and metonitazene was utilized as the standard was previously prepared. The first parameter that was adjusted was the run time. Utilizing the steroid method as a template, the oven program and temperature stayed the same, however the final hold at 280°C for 19 minutes, was reduced to 9 minutes, resulting in a run time of 15 minutes. Most of the nitazene analogs have a retention time of about 11 minutes, which allowed for a much shorter method and improved method speed. Next, to increase the abundance of the peaks on the mass spectrum, the split ratio was adjusted from 20:1 to 10:1. However, this did not change the mass spectra appreciably, so instead the split ratio was set back to 20:1 and the injection volume was increased from 0.2 µL to 1 µL. This

change did have a positive impact on the mass spectrum, as well as the chromatogram. However, there were extraneous peaks, compounds often seen as a result of column bleed, showing up on the chromatogram due to overabundance, so the split ratio was decreased to 30:1. The final parameters for the nitazene method were a split ratio of 30:1, 15-minute length, a split flow of 30 mL/min, and an injection volume of 1 µL. At the end of the method development, all of the nitazenes were added to the instrument's internal libraries.

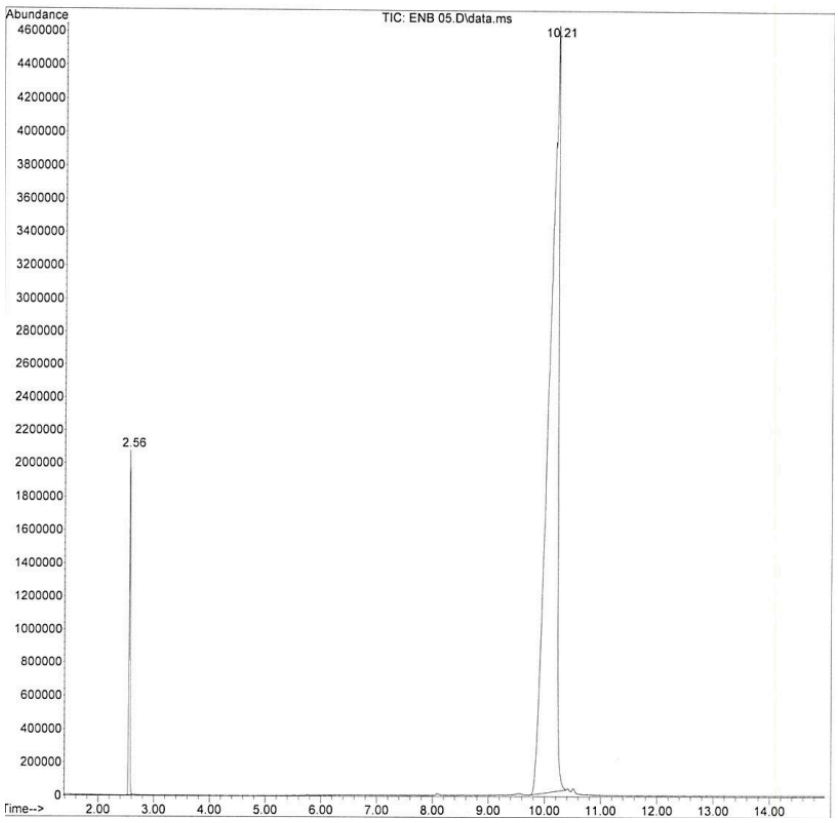


Figure 21 *Chromatogram of metonitazene using the nitazene method.*

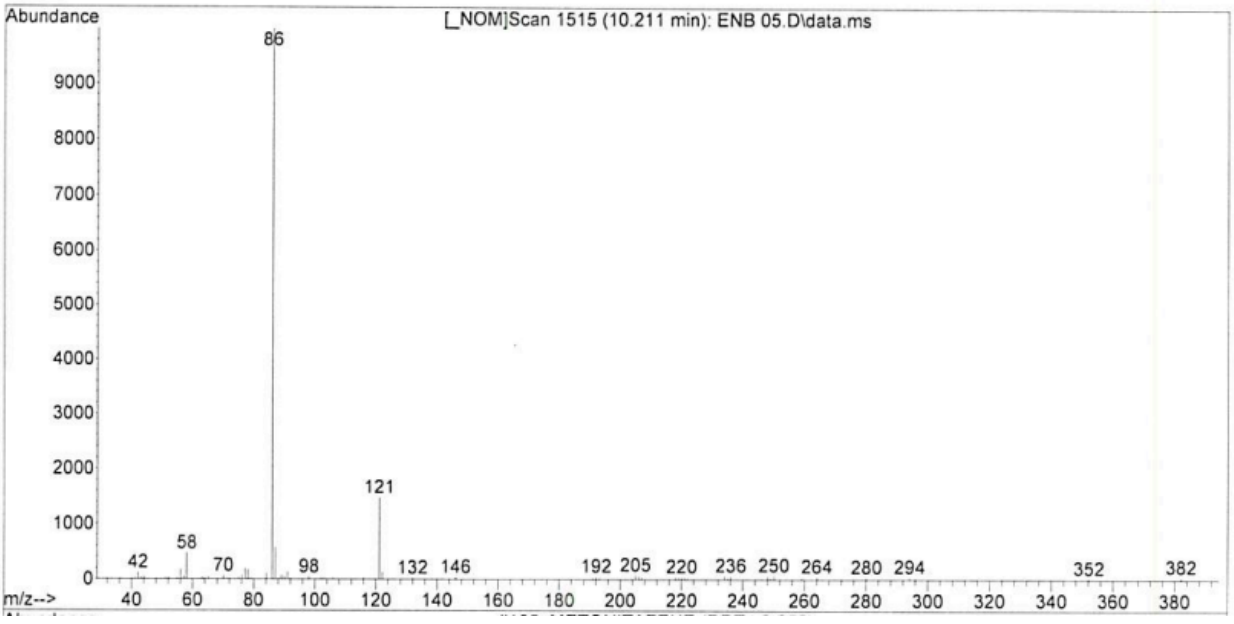


Figure 22 *Mass spectrum of metonitazene using the nitazene method.*

ATR-FTIR

Nitazene citrate, isotonitazene, and metonitazene were attempted to be analyzed by FTIR with the ATR attachment. The ATR-FTIR spectra were processed using the standard ATR effective pathlength correction, which facilitates inclusion and searching of both ATR and transmission spectra in a single library. Spectra were obtained using a La-DTGS detector with co-addition of 64 scans per spectrum.

Since nitazene citrate and isotonitazene were in powder form, the ATR-FTIR was able to successfully obtain characteristic spectra, however, because the metonitazene was in acetonitrile, the ATR-FTIR spectra were only characteristic of the solvent and not the drug itself. As a result, only isotonitazene and nitazene citrate were added to the FTIR internal library. A future study could try evaporating the solvent away from the metonitazene and analyze the powder that is left.

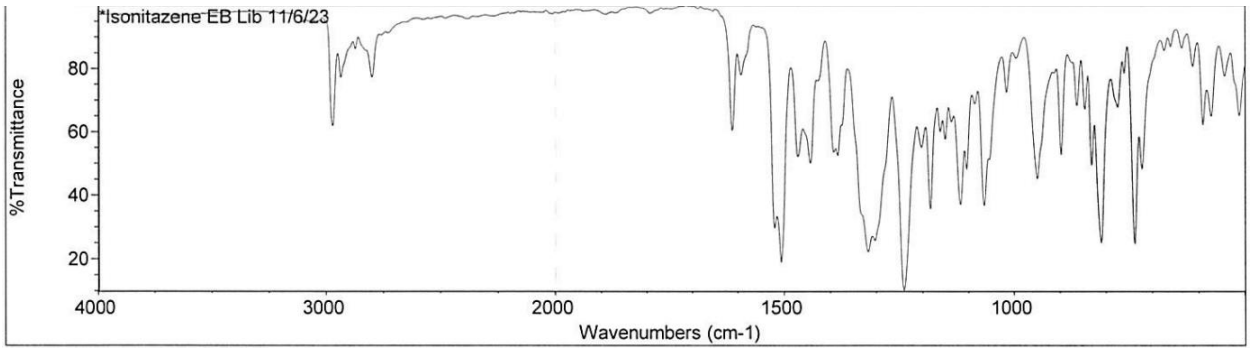


Figure 23 *FTIR spectrum of isotonitazene.*

Figure 23, shows the ATR-FTIR corrected spectrum for neat solid isotonitazene powder. The variety of sp^2 and sp^3 hybridized C-O, C-N, and N-O moieties leads to strong transitions with rich structure spanning the range from about 1700 to 700 cm^{-1}.

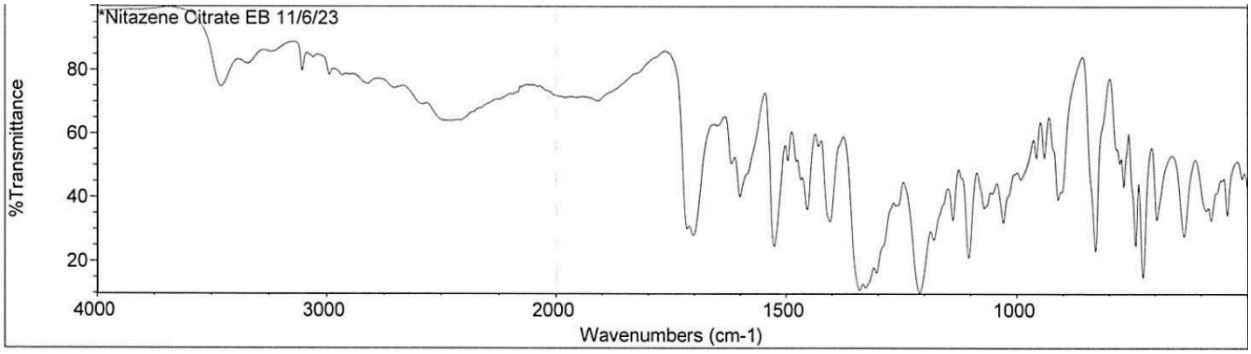


Figure 24 *FTIR spectrum of nitazene citrate.*

Figure 24 shows the AT-FTIR spectrum for neat, solid nitazene citrate powder. It shows similar rich structure below ~1700 cm^{-1}, but also has significant broad structures between 1700 and 3800 cm^{-1}.

TruNarc

The last method of analysis performed on these three analogs was the handheld Raman spectrometer, TruNarc. This is a device that many police officers and prison guards use to presumptively test drugs. It is speculated in the future that this method will replace color tests. Before testing the suspected drugs, a check of the laser is performed by identifying a polystyrene cover that comes attached to the instrument. Once that is done, the laser is simply pointed at the drug of interest and held for about 2 minutes. The result will come back as controlled, a precursor, not controlled, or inconclusive. When these three nitazenes were first scanned on the TruNarc the results came back as inconclusive because these compounds were not in the internal library of the instrument. Once the library was updated to the newest version, some of the nitazene analogs were added and isotonitazene was identified. Nitazene citrate was not added to

the new library and still showed up as inconclusive and because metonitazene was in an acetonitrile solution, the TruNarc only identified the acetonitrile

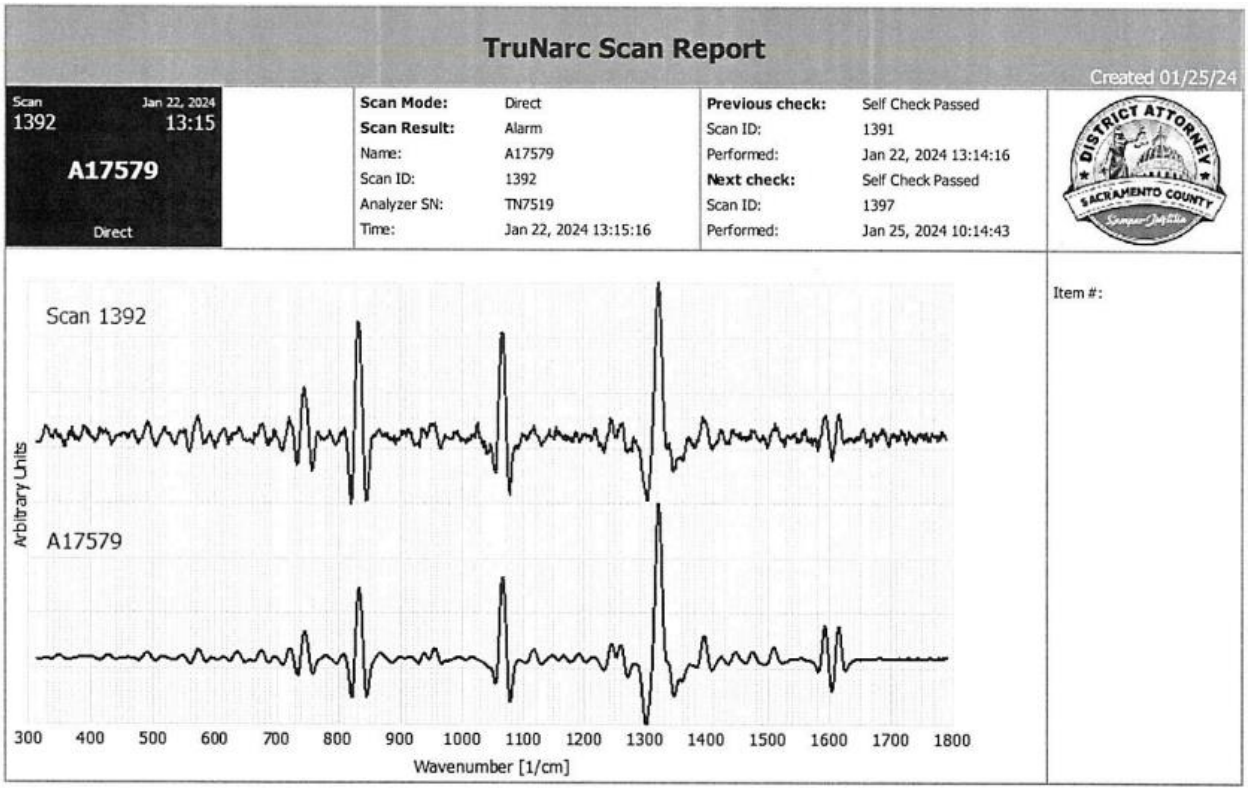


Figure 25 *Spectrum match on the TruNarc for isotonitazene.*

Nitazene citrate, isotonitazene and metonitazene were all characterized to the best of the lab's ability and with the development of the nitazene method on the GC-MS, the criminalists are now able to be on the lookout for these novel synthetic opioids in their future casework whether they use the FTIR, the TruNarc, color tests, or the GC-MS.

Chapter 4: Results for Protonitazene, Etonitazene, and N-Pyrrolidino Etonitazene (etonitazepyne)

The last three standards that were tested were protonitazene, etonitazene, and etonitazepyne. Etonitazene is the most potent of the nitazene analogs and is one of the most common one to be abused and many other drugs are "cut" with it. That being said, color tests were not able to be done on etonitazene and protonitazene because the amount received was only 5 mg. Unfortunately, there was not enough standard to conduct all the color tests, FTIR, and GC-MS determination. As a result, only color tests and extraction for GC-MS were chosen.

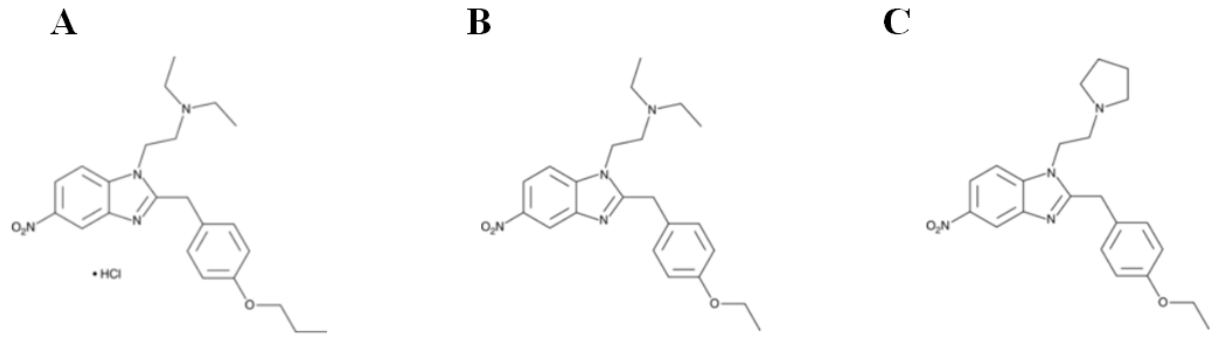


Figure 26 *Structures of analogs studied in this section. A) protonitazene B) etonitazene C) etonitazepyne*

Color Tests

Interestingly, etonitazepyne was not originally going to be characterized in this study, however, the lab had a standard of it that they did not realize they had and graciously allowed its use for this project because they had not characterized it themselves yet. Color tests were able to be done on the etonitazepyne, because there was 5 mg in 5 mL of methanol. One mL was used for the color tests and 0.5 mL was used for the extraction and GCMS analysis.

Figure 27 *Color tests for etonitazepyne. Ferric Chloride is not shown here. 1= Marquis, 2= Nitroprusside, 3= cobalt thiocyanate, 4= ferric chloride (not shown), 5= Mecke, 6= Froehde, 7= Van Urk, 8= Wagner, 9= Dille-Koppanyi, 10= Lierbermann's.*

The etonitazepyne had similar color test results to metonitazene, however it did not react with the nitroprusside, cobalt thiocyanate, or Mecke. It also had a different color change for the Liebermann's, turning a gray green and bubbling rather than turning brown like the others. These differences are likely due to the extra pyrrolidino ring on the base structure changing the way the reagents react with the drug. This is a similar reaction that the synthetic cathinones 2C-B and 2C-D have with the Liebermann's reagent. They have similar ether functional groups as well as benzene rings.

Extraction and GCMS Results

Base extraction was used for all three of these analogs because it was proven to be the most effective extraction method when tested on nitazene citrate, isotonitazene, and metonitazene. Due to the limited quantities of these compounds, other extraction procedures were not assessed.

These analogs had a similar retention time as isotonitazene and metonitazene, so they were run on the steroid method first to get a better understanding of retention times and to determine if the developed nitazene method developed would be suitable for analysis. The first analyzed was etonitazene and it came out around 11 minutes and was a good candidate for the nitazene method. It had a similar fragmentation pattern to isotonitazene, with major ions at 86 m/z and 107 m/z. These ions are used to differentiate this analog from the others.

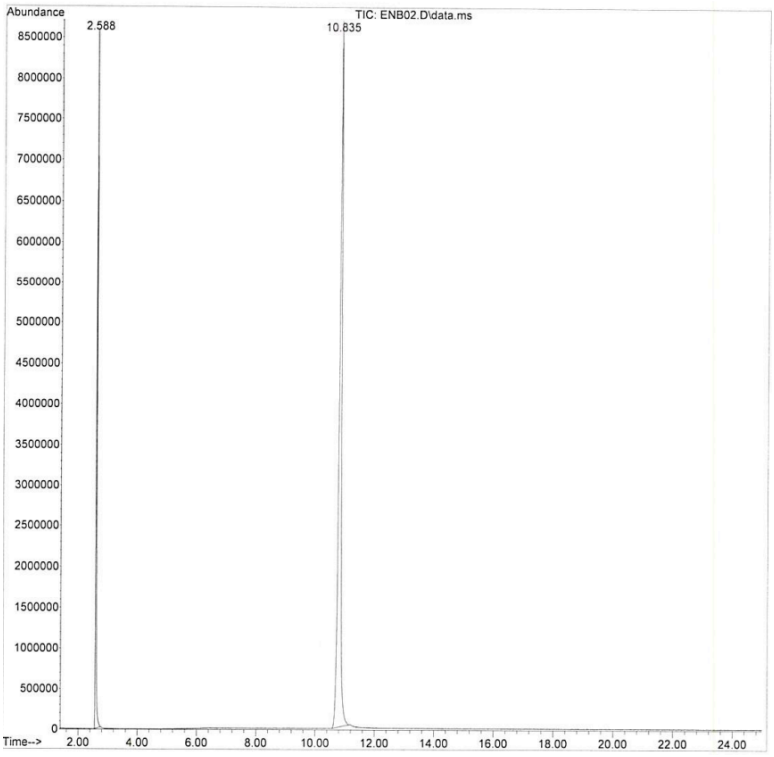


Figure 28 *Chromatogram of etonitazene (10.835 min) using the steroid method.*

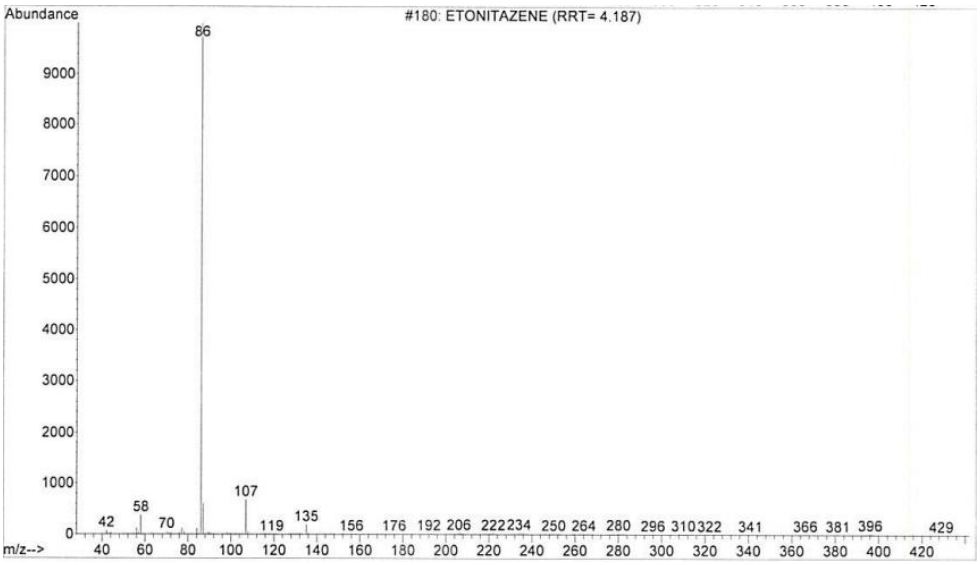


Figure 29 *Mass spectrum of etonitazene.*

The next analog analyzed was protonitazene, which had a retention time of about 12 minutes and was also a good candidate for the nitazene method. It also had a fragmentation pattern similar to isotonitazene with major ions at 86 m/z and 107 m/z.

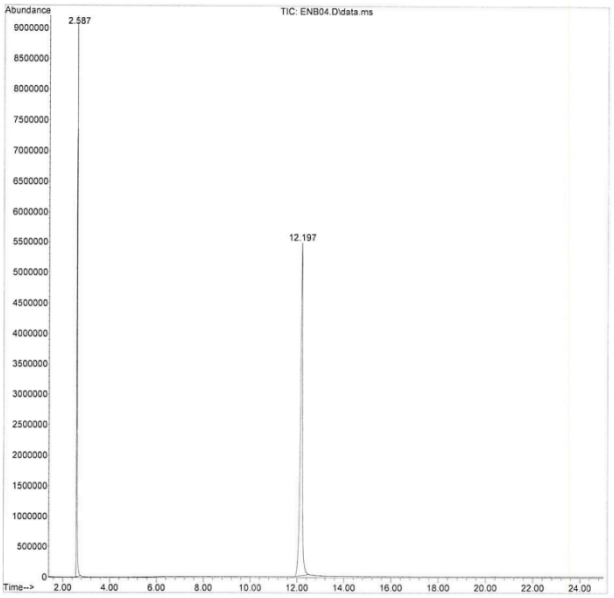


Figure 30 *Chromatogram of protonitazene using the steroid method.*

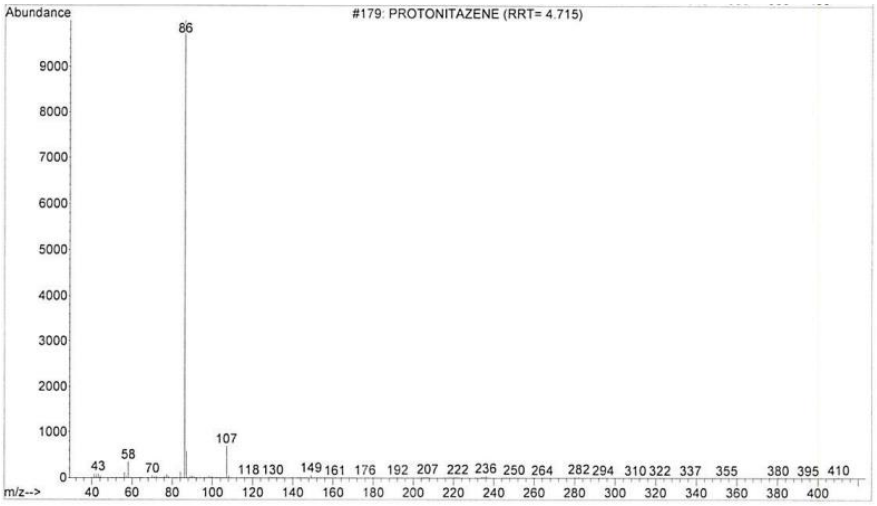


Figure 31 *Mass spectrum of protonitazene.*

The last analog in this section is the etonitazepyne. During analysis, there were several issues with the abundance on the chromatogram. It never exceeded one million, which is not typical of the nitazenes that have been tested so far. This standard was much older than the others and may have degraded over time. The lab does not have policies concerning how long standards are kept and these were all kept in the freezer. However, it was still usable for characterization and for analysis. Etonitazepyne had a retention time of about 15 minutes when run on the steroid method. Due to other analyst use on Instrument 9, etonitazepyne could not be run on the nitazene method, and had to be run on Instrument 11 on the steroid method. The fragmentation of the etonitazepyne is slightly different than the other analogs with major ions at 84 m/z, 107 m/z, and 207 m/z. The 107 m/z ion is likely the same as the other analogs, but it is interesting that there is a major ion at 84 m/z instead of 86 m/z. Again, these ions were interesting to observe, but were only really used to confirm the identity of this analog during the library match.

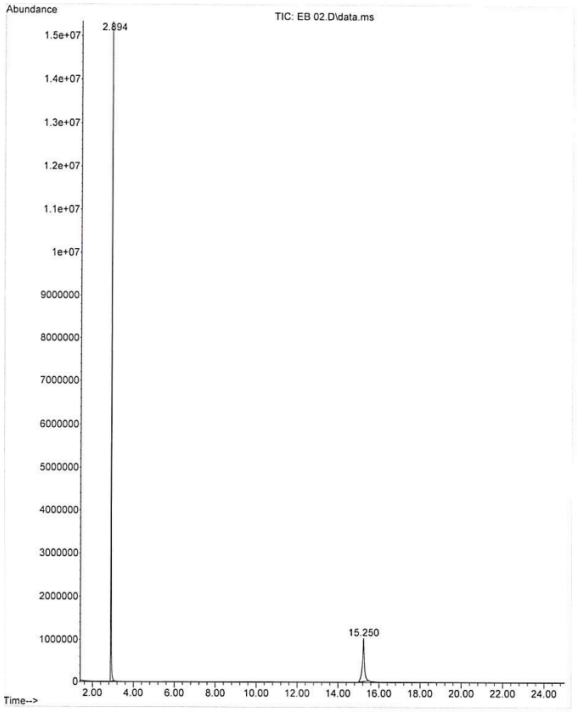


Figure 32 *Chromatogram of etonitazepyne using the steroid method.*

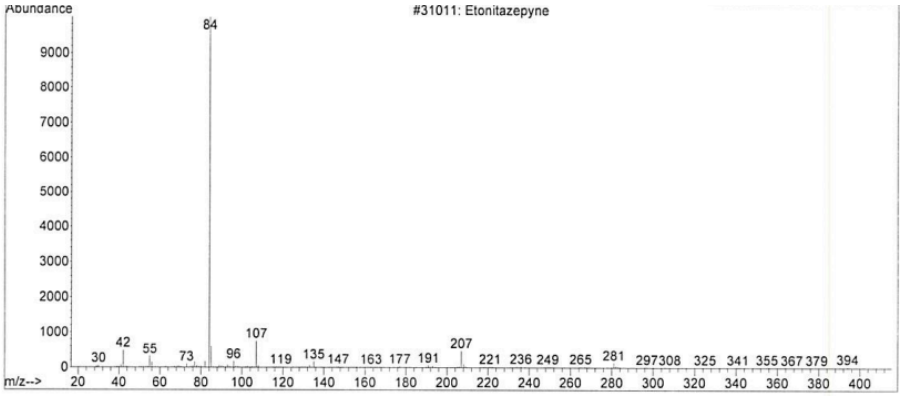


Figure 33 *Mass spectrum of etonitazepyne.*

FTIR

The only standard of these three that was able to be analyzed by ATR-FTIR was protonitazene. There was not enough of the etonitazene to be analyzed after the extraction and etonitazepyne was in a solution of methanol so the ATR-FTIR would only detect the methanol and not the drug itself. Again, a future study could evaporate the solvent and analyze the etonitazepyne using ATR- FTIR. There was enough protonitazene for ATR-FTIR analysis and the results shown in Figure 34.

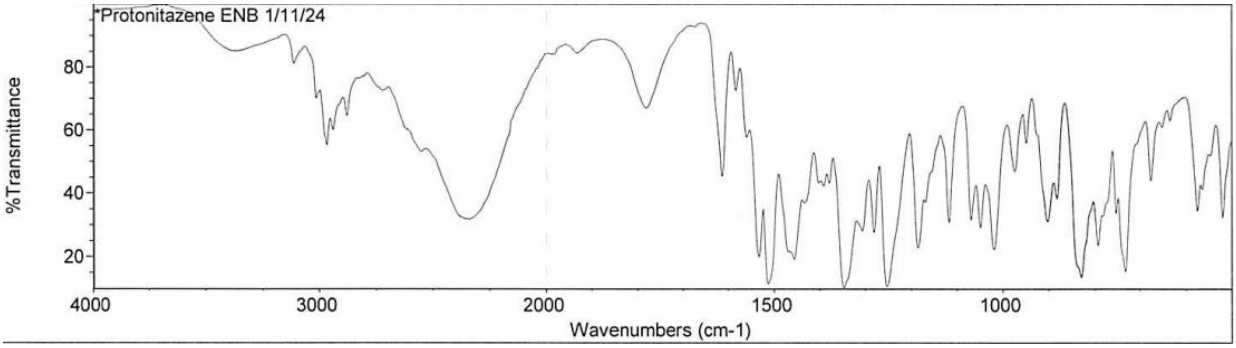


Figure 34 FTIR spectrum of protonitazene.

The ATR-FTIR spectrum for etonitazepyne exhibits similar general features as the nitazene citrate and the isotonitazene, with rich, narrow peak structures below 1700 cm^{-1}, and a mixture of narrow and broad bands above. The three ATR-FTIR spectra obtained are consistent with those for several nitazene derivatives from Kimani et al.

TruNarc

It was not possible to use the TruNarc on these compounds because there was not enough etonitazene and protonitazene for the instrument to sample. As with metonitazene, the etonitazepyne was in solution, so the instrument only registered the methanol. Again, a future study could remedy this by evaporating the solvent.

Now that these three analogs were added into the internal libraries of the instruments, counterfeit pills pulled from casework were able to be analyzed for the presence of illicit substances including the nitazenes.

Chapter 5: Results for Counterfeit Pills

Four types of counterfeit pills were analyzed for this study. Three were from an FBI case (Case number: 23-002716 Items 52-54) that had thousands of counterfeit pills as well as a host of other drugs. The fake oxycodone pills were taken from a separate case in El Dorado Hills, CA and used as a standard in the lab for fentanyl in counterfeit pills. They were all tested for the presence of nitazenes as possible contaminating analytes. Counterfeit oxycodone pills are a popular way for nitazenes to be administered and should be tested.

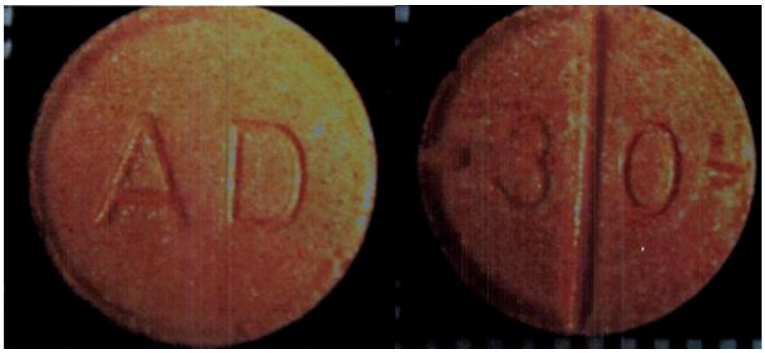


Figure 35 *Item 54, the pill labeled as N23 front and back.*

Figure 36 *Item 53, labeled as N24 front and back.*

Figure 37 *Item 52, labeled N25 front and back.*

Figure 38 *Counterfeit oxycodone pills, labeled F13, front and back.*

Table 3 *Color test results for counterfeit pills.*

	Marquis	Nitro-prusside	Co(SCN)2/ SnCl2	Ferric Chloride	Mecke	Froehde	Van Urk	Wagner	Dille-Koppanyi	Liebermann's
Steps	1. H2SO4 2. Formald.	1.Nitroprusside 2. Saturated Sod.Carbonate	1. Cobalt Thiocyanate 2. Stannous Chloride	1. Ferric Chloride 2. DI water	1. Selenious acid 2. Sulfuric Acid	1. Molybdic acid 2. Sulfuric Acid	1. Van Urk reagent 2. Con. HCl	1. 0.1N HCl 2. Wagner reagent	1. Rgt A 2. Rgt B	1. Potassium nitrite 2. Sulfuric acid
N23	No reaction	No reaction	Blue specks	No reaction	No reaction	No reaction	Yellow	Orange brown	No reaction	No reaction
N24	Black	Dark blue	Blue specks	No reaction	Teal green	Black	Yellow	Shiny brown precip	No reaction	Black
N25	Green/purple	Blue	Blue specks	No reaction	Teal green	Black	Yellow	Shiny brown precip	No reaction	Black
F13	No reaction	No reaction	Blue specks	No reaction	No reaction	Blue/green	Yellow	Shiny brown precip	No reaction	Brown specks

N23, N24, and F13 were counterfeit pills containing fentanyl, and its precursor 4-ANPP and N25 was found to contain MDMA. However, these samples via the standard drug method and any possible nitazenes that were in these pills would have gone unnoticed so it was interesting to see if they also contained nitazenes that may have been overlooked. First, color tests were performed on the pills and compared to the nitazene standard color tests. N23 and F13 had results that were consistent with what is typically seen when fentanyl is present and did not react in the same way as the nitazenes. N24 had some of the same reactions as the nitazenes with the Marquis test and Wagner, however the rest were dissimilar and were likely consistent with fentanyl and 4-ANPP and possibly caffeine. N25 had the same Wagner results as N24 likely indicating the presence of caffeine. The Marquis test was positive for MDMA and the rest of the reactions were likely due to other binders in the pill and are not necessarily indicative of other drugs present.

Figure 39 *N23 color tests. 1= Marquis, 2= Nitroprusside, 3= cobalt thiocyanate, 4= ferric chloride (not shown), 5= Mecke, 6= Froehde, 7= Van Urk, 8= Wagner, 9= Dille-Koppanyi, 10= Lierbermann's.*

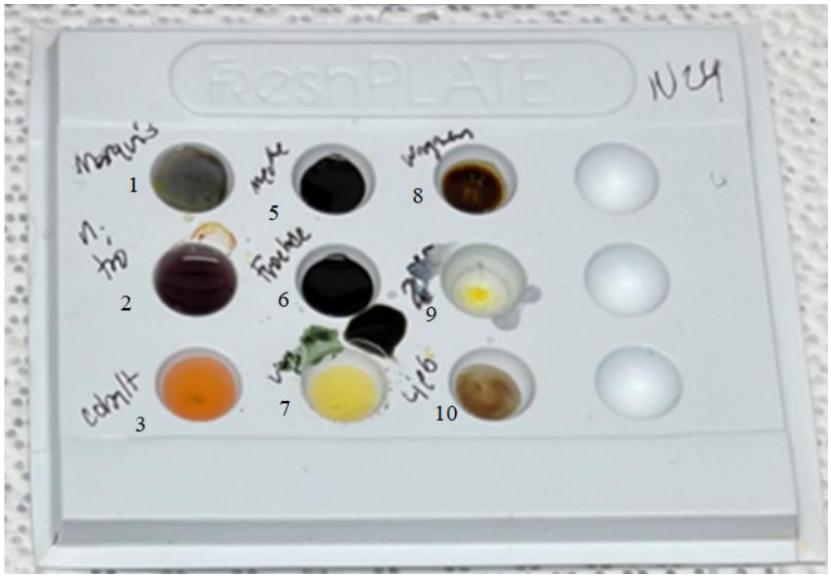


Figure 40 *N24 color tests. 1= Marquis, 2= Nitroprusside, 3= cobalt thiocyanate, 4= ferric chloride (not shown), 5= Mecke, 6= Froehde, 7= Van Urk, 8= Wagner, 9= Dille-Koppanyi, 10= Lierbermann's.*

Figure 41 *N25 color tests. 1= Marquis, 2= Nitroprusside, 3= cobalt thiocyanate, 4= ferric chloride (not shown), 5= Mecke, 6= Froehde, 7= Van Urk, 8= Wagner, 9= Dille-Koppanyi, 10= Lierbermann's.*

Figure 42 *F13 color tests. 1= Marquis, 2= Nitroprusside, 3= cobalt thiocyanate, 4= ferric chloride (not shown), 5= Mecke, 6= Froehde, 7= Van Urk, 8= Wagner, 9= Dille-Koppanyi, 10= Lierbermann's.*

After doing the color tests, the pills were run on the nitazene method to determine if any nitazenes were missed in routine analysis. They were all extracted using base extraction and methanol dissolution and about half of a pill was used for each extraction method. Methanol dissolution ended up being the best extraction method. This was because the goal of the extraction had changed from just wanting to get a specific compound to extracting as many compounds as possible, as it is unknown what the pills were made up of. N23, N24, N25 had to be rerun a few times because the drugs that they were initially thought to contain did not show up on the chromatogram. After trying a few times, they were run on the drug method to ensure that these pills did have the drugs indicated which was successful. They did not show up on the runs using the nitazene method because it was based off the steroid method, so the solvent delay covered the area in which the drugs would elute. The drug method has different parameters, so the solvent delay does not impact drugs that come out at earlier retention times. There were no problems analyzing the F13 pills. Unfortunately, none of the pills were found to have any nitazene analogs. There were no peaks on the chromatograms past 10 minutes in the range that the nitazenes would come out using the nitazene method.

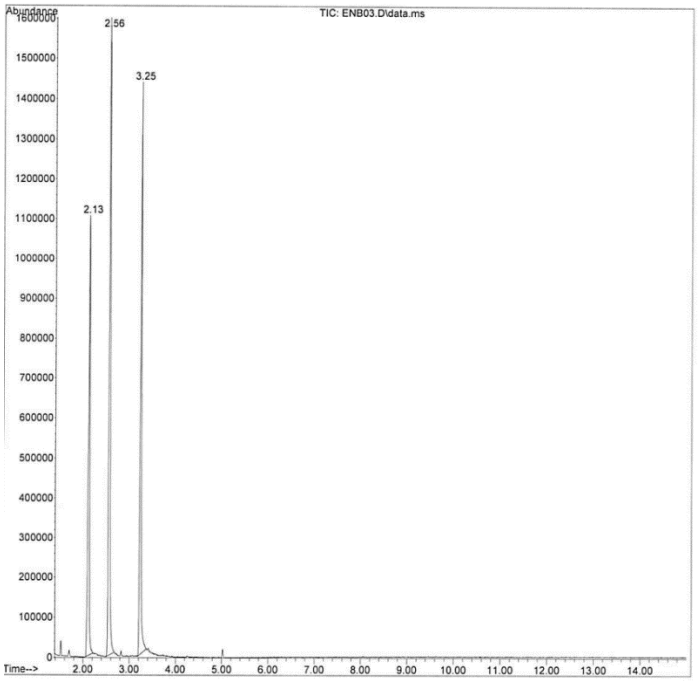


Figure 43 *Chromatogram of N23 with methanol dissolution using the nitazene method.*

N23 was found to contain n-hexadecanoic acid, internal standard, and 1-heptadecanecarboxylic acid when run using the nitazene method. It was found to contain methamphetamine, n-hexandecanoic acid, internal standard, and octadecanoic acid using the drug method. Interestingly, though methamphetamine was found, that was not the drug that was originally found in this case, indicating that these pills could have a mixture of drugs in them and not just one. All mass spectra for the compounds found in these runs can be found in the Appendix.

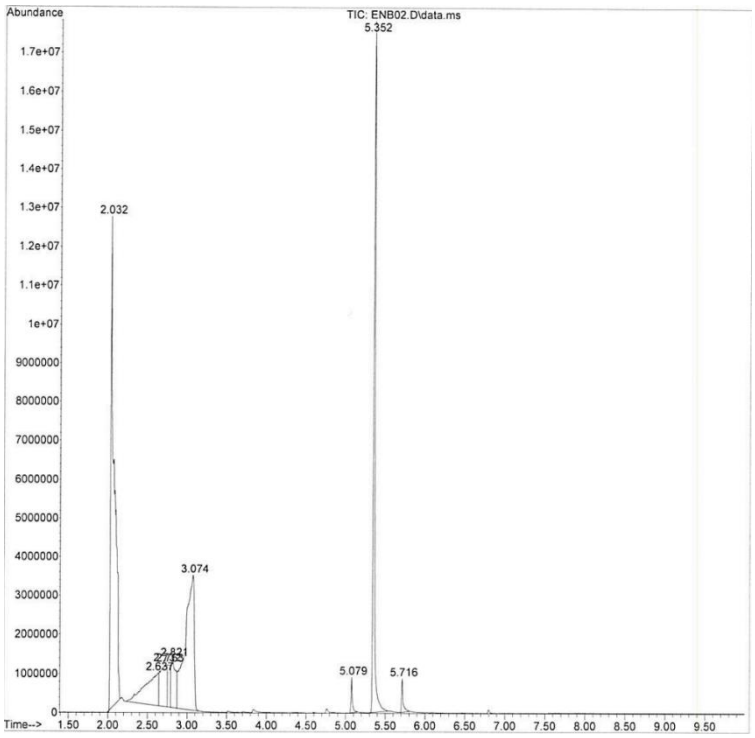


Figure 44 *N23 chromatogram with methanol dissolution when run on the drug method.*

N24 was found to contain caffeine, n-hexadecanoic acid, internal standard, octadecanoic acid, and MDMA when run on the nitazene method with methanol dissolution. When run on the drug method, N24 was found to contain MDMA, methedrone, caffeine, n-hexadecanoic acid, octadecanoic acid, and internal standard. Again, when using the nitazene method, there are no notable peaks above 10 minutes.

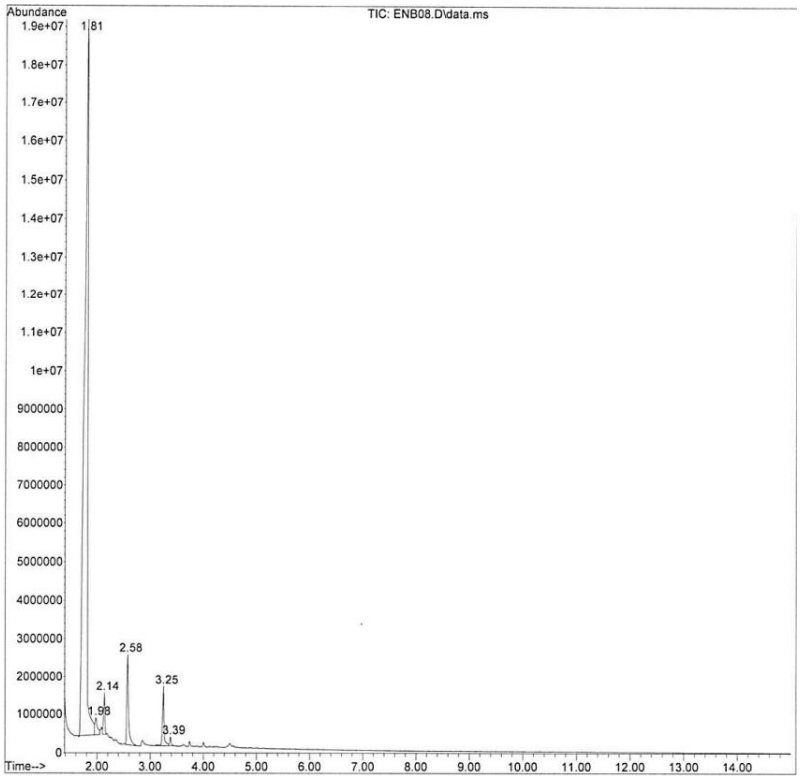


Figure 45 *N24 chromatogram with methanol dissolution run on the nitazene method.*

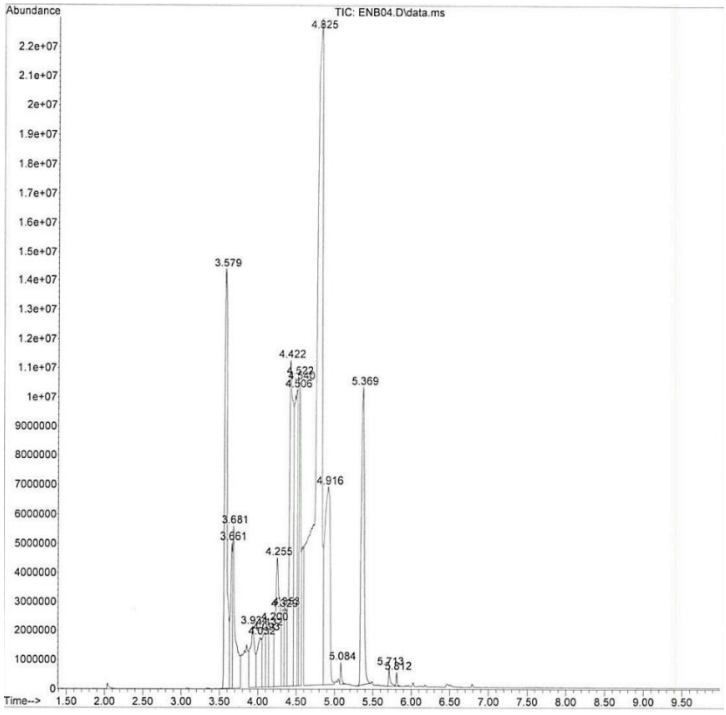


Figure 46 *N24 chromatogram with methanol dissolution run on the drug method.*

N25 was found to contain caffeine, internal standard, octadecanoic acid, 4-methoxy-4-methyl-8-oxibicyclo[3.2.1]oct-6-en-3-one, and methedrone when run using methanol dissolution and the nitazene method. When run on the drug method and using methanol dissolution, N25 was found to contain MDMA, 4-methylcathinone, methedrone, caffeine, n-hexandecanoic acid, octadecanoic acid, 4-cyclopentene-1,2,3-trione, 4,5-dihydroxy-, and 6,7-dimethyl-diazabucyclo[3.2.1]nonan-3-one. This pill seems to be the only one of these three that had what it was originally reported to have in it. Once again there are no significant peaks past 10 minutes where the nitazenes would be.

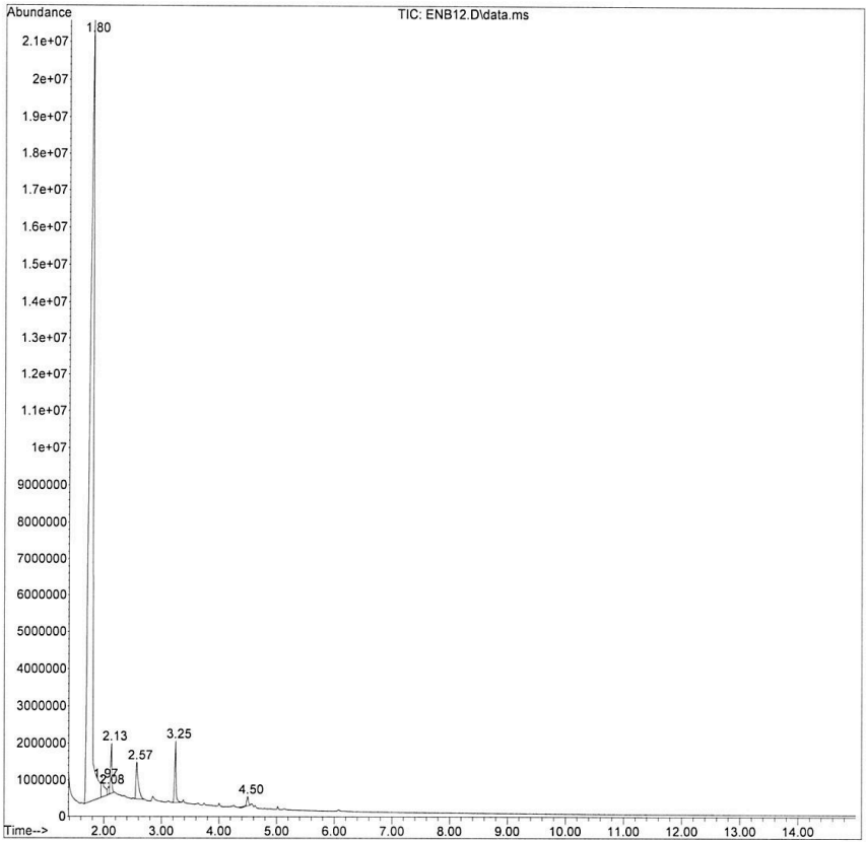


Figure 47 *N25 chromatogram using methanol dissolution and the nitazene method.*

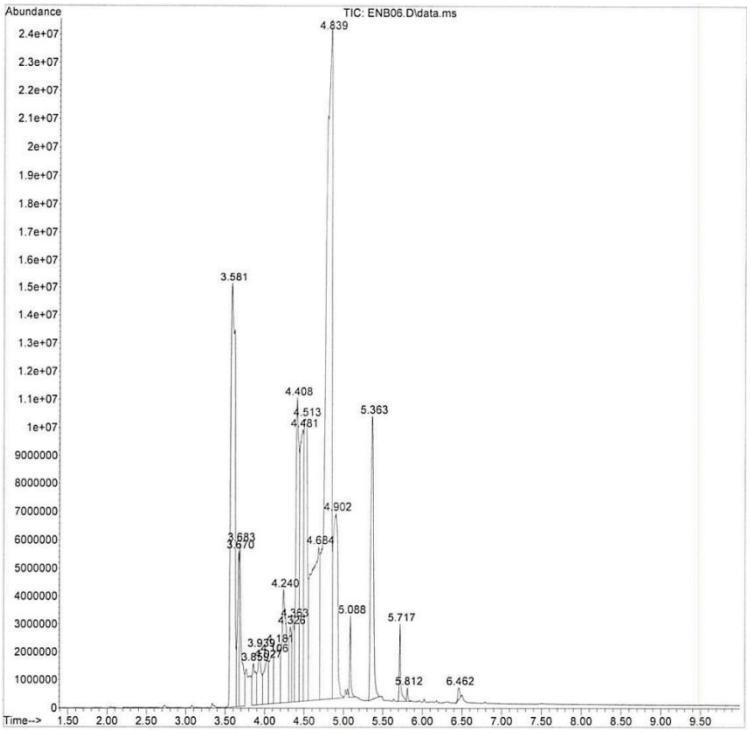


Figure 48 *N25 chromatogram using methanol dissolution and the drug method.*

F13 contained fentanyl and 4-ANPP when run on the nitazene method, so there was no need to rerun this pill using the drug method because it was very clear that there were illegal drugs present that were not nitazenes.

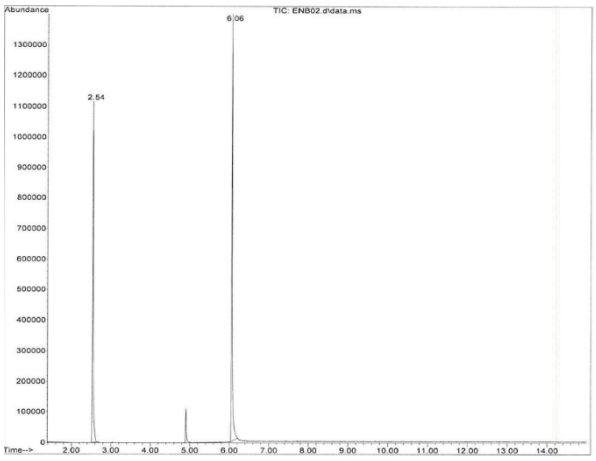


Figure 49 *F13 chromatogram using base extraction on the nitazene method.*

Although there were no nitazenes found in these pills, this does not mean that there are no nitazenes in California or Sacramento, it just means that these particular cases did not have them. There have been several documented cases mentioned in the background section that prove that nitazenes are moving westward across the United States and it is still important for labs to look for them in their counterfeit pill cases.

Conclusion

This project set out to characterize nitazene analogs because they are moving west across the United States and crime labs need to be prepared to deal with them. Fentanyl had a similar start and now has turned into the most widely seen illicit substance besides marijuana and alcohol. Knowing as much as possible about these nitazenes is essential for the preparation of labs all across the country so that when they surge like fentanyl did, labs will be ready. The color tests allow analysts to have an idea of the type of drug they are dealing with and the method created here is easy to apply when nitazenes are suspected. This project resulted in all of the nitazene analogs tested to be added to the instrumental libraries, so even if the nitazene method is not used, they can still be detected. Though, this was not an entirely novel idea, it was new to this crime lab and benefitted them greatly. There are other crime labs on the west coast that may not be entirely aware of the new nitazenes and they will be a significant disadvantage when overdose cases involving nitazenes come in.

This project had several limitations, however. The color tests are only presumptive and as a result, they have a lot of overlapping results with more common seized drugs. The nitazene method, though sufficient for the analogs recorded here, may not be suitable for all of the nitazene analogs and could be modified. In the future, there will be other analogs of nitazenes that will need to be characterized just as these were to keep up with the loopholes that drug creators will find. Future projects could also expand the instrumentation and add additional analogs to the TruNarc library so that police officers and prisons can identify these drugs at the point of use. Nitazenes are dangerous and as a result of this project the Sacramento District Attorney's Laboratory of Forensic Services is ready to detect them.

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Appendix A

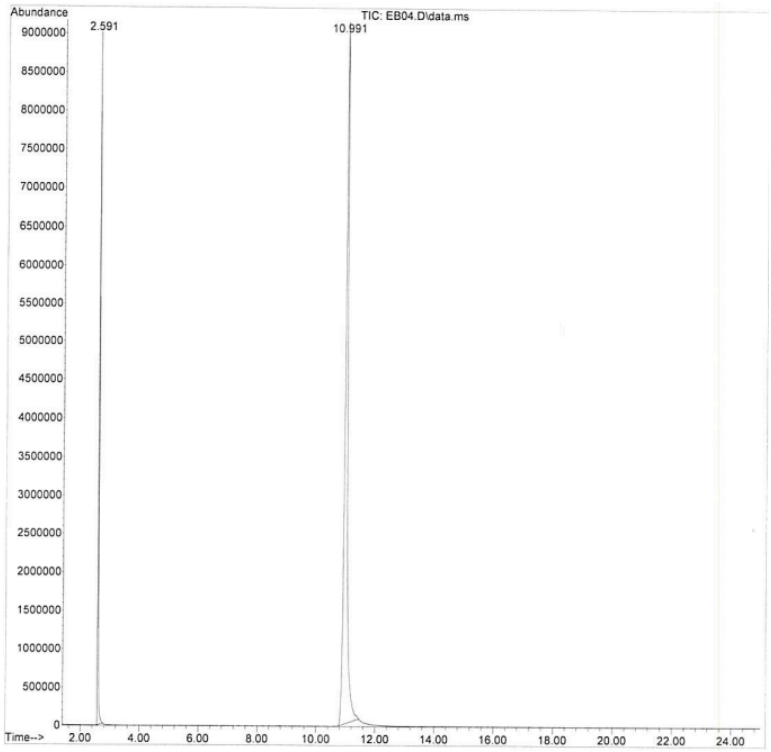

Figure A1 Chromatogram of isotonitazene using base extraction on steroid method.

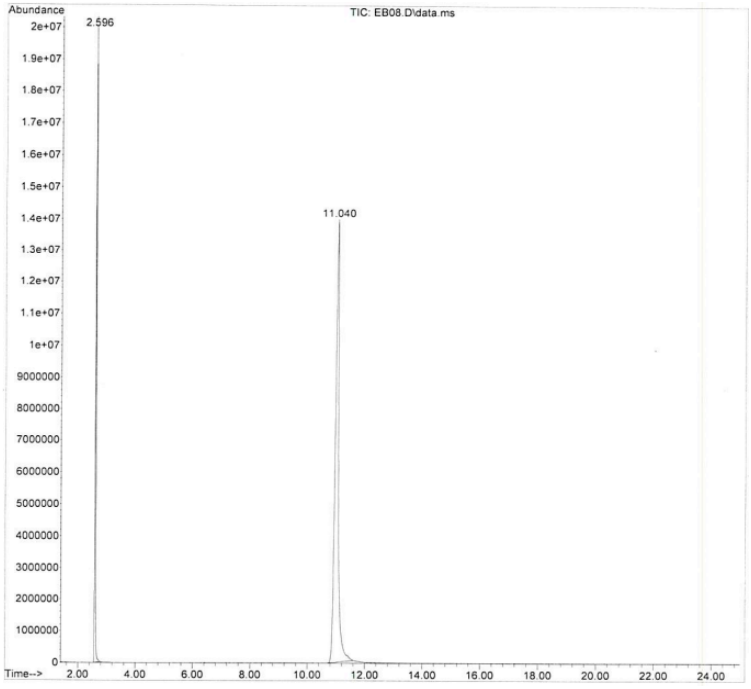

Figure A2 Chromatogram of isotonitazene using pH 9 extraction on steroid method.

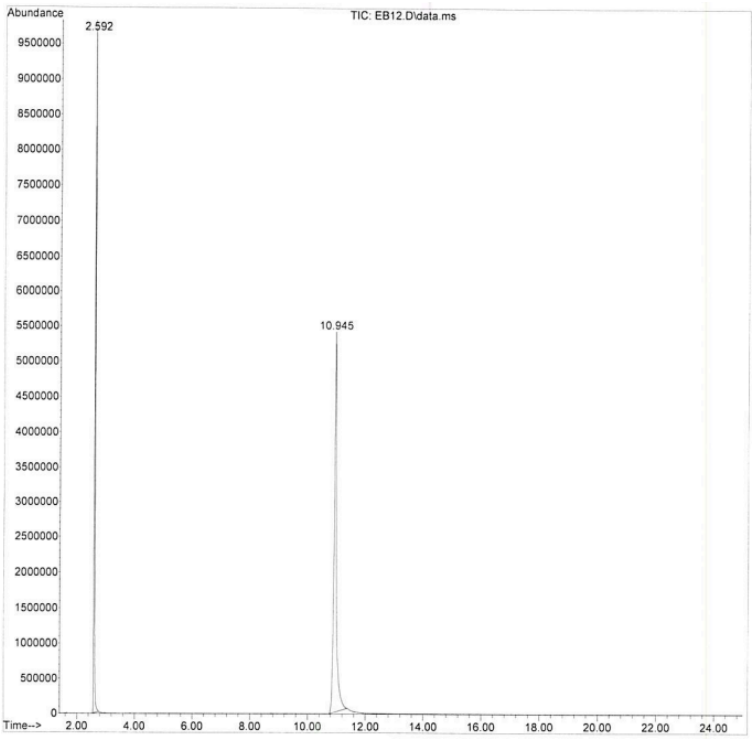

Figure A3 Chromatogram of isotonitazene using methanol dissolution on steroid method.

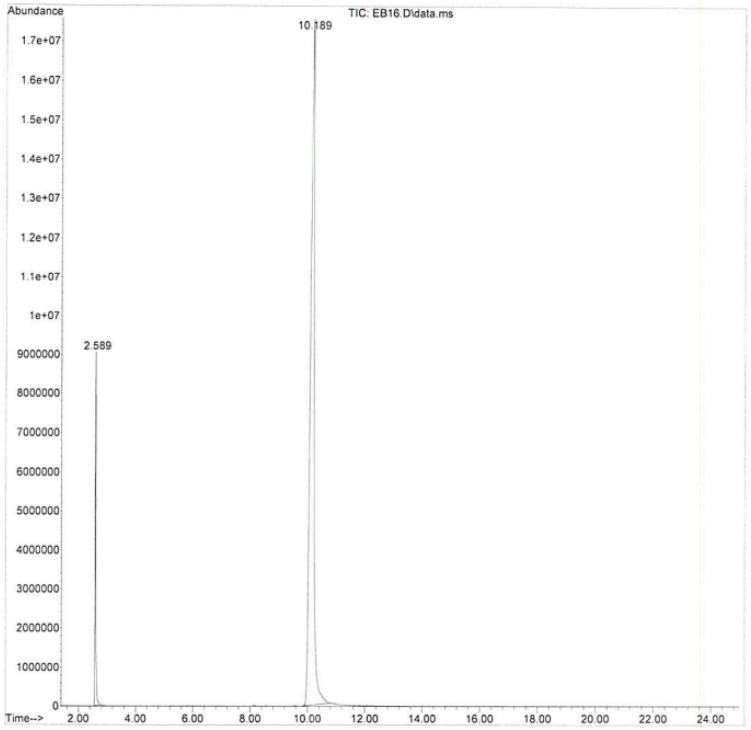

Figure A4 Chromatogram of metonitazene using base extraction on steroid method.

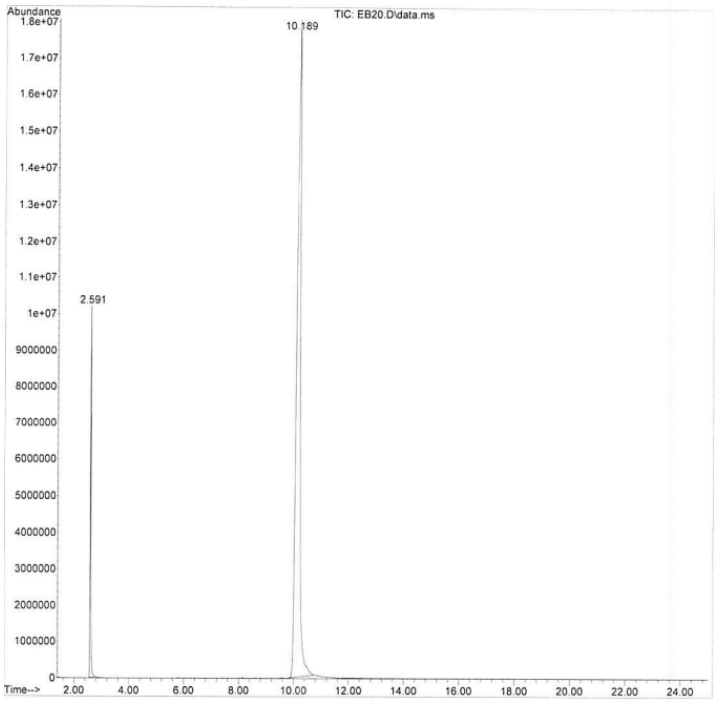

Figure A5 Chromatogram of metonitazene using pH 9 extraction on steroid method.

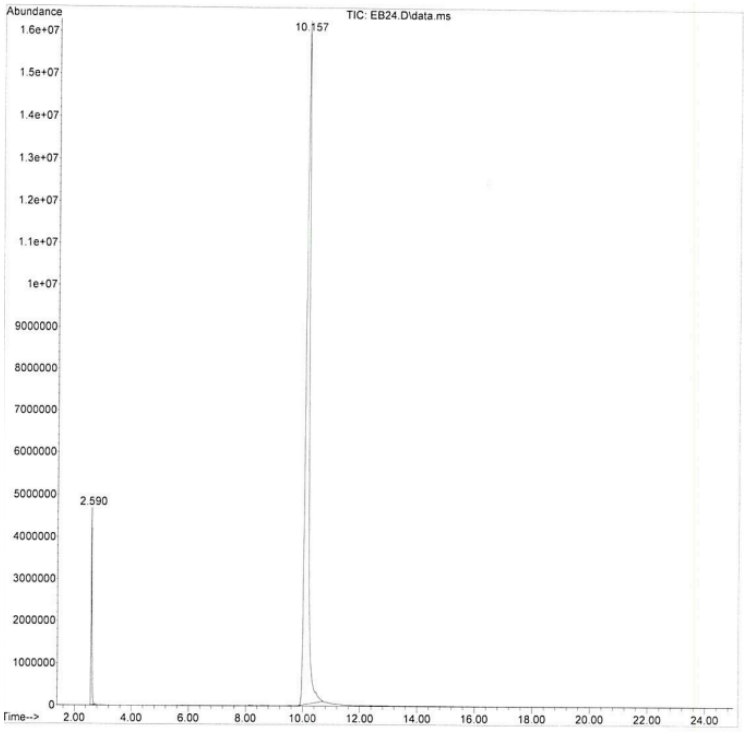

Figure A6 Chromatogram of metonitazene using methanol dissolution on steroid method.

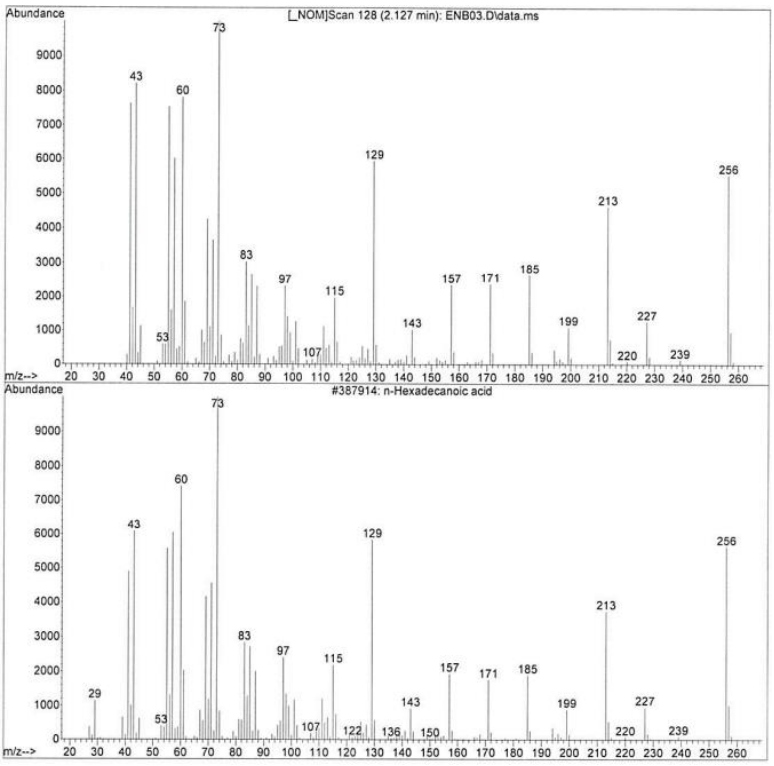

Figure A7 Mass spectrum of n-Hexadecanoic acid in N23 using the nitazene method. The bottom spectrum shows the library match.

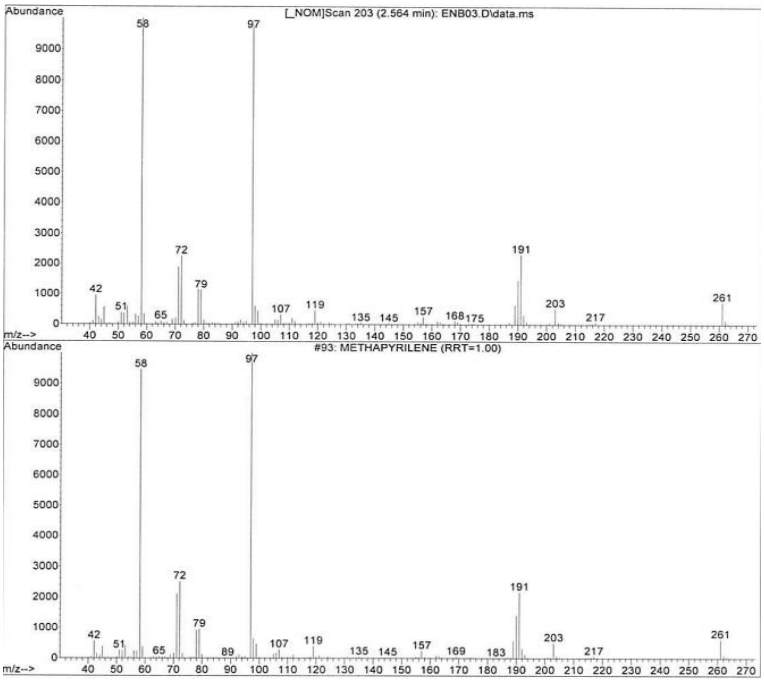

Figure A8 Mass spectrum of internal standard in N23 using the nitazene method. The bottom spectrum shows the library match.

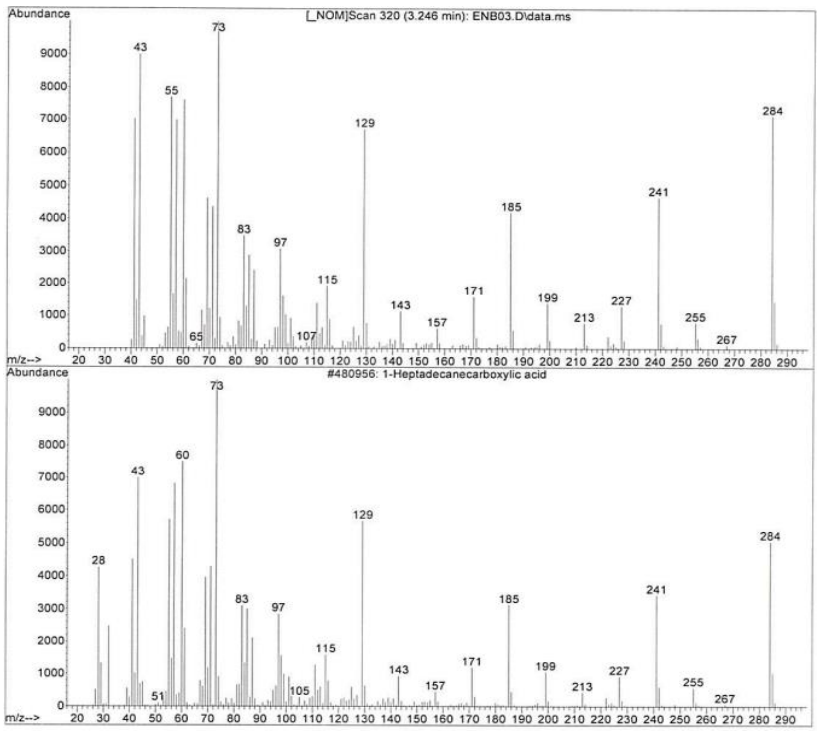

Figure A9 Mass spectrum of n-Heptadecanecarboxylic acid in N23 using the nitazene method. The bottom spectrum shows the library match.

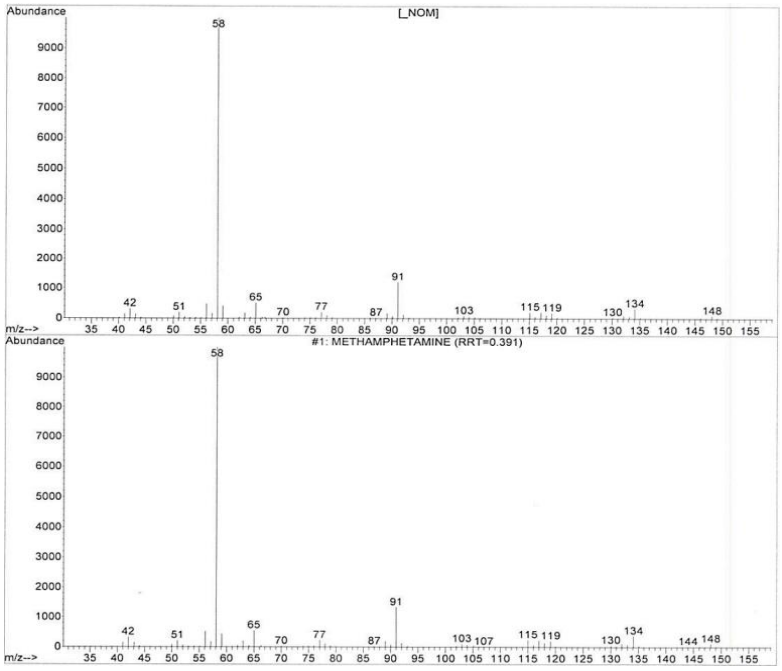

Figure A10 Mass spectrum of methamphetamine in N23 using the drug method. Methamphetamine accounts for the peaks from 2.034 minutes to 3.073 minutes. The bottom spectrum shows the library match.

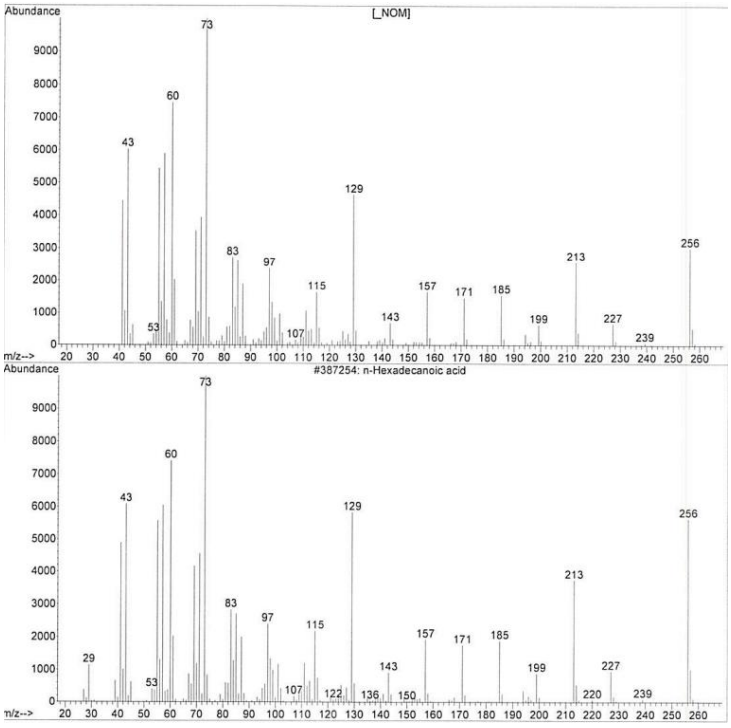

Figure A11 Mass spectrum of n-Hexadecanoic acid in N23 using the drug method. This accounts for the peak at 5.077 minutes. The bottom spectrum shows the library match.

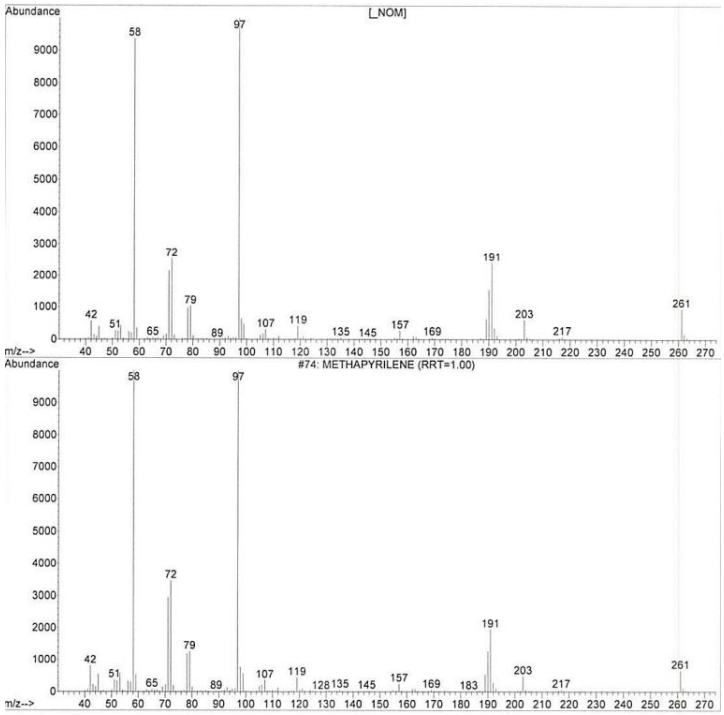

Figure A12 Mass spectrum of internal standard in N23 using the drug method. This accounts for the peak at 5.354. The bottom spectrum shows the library match.

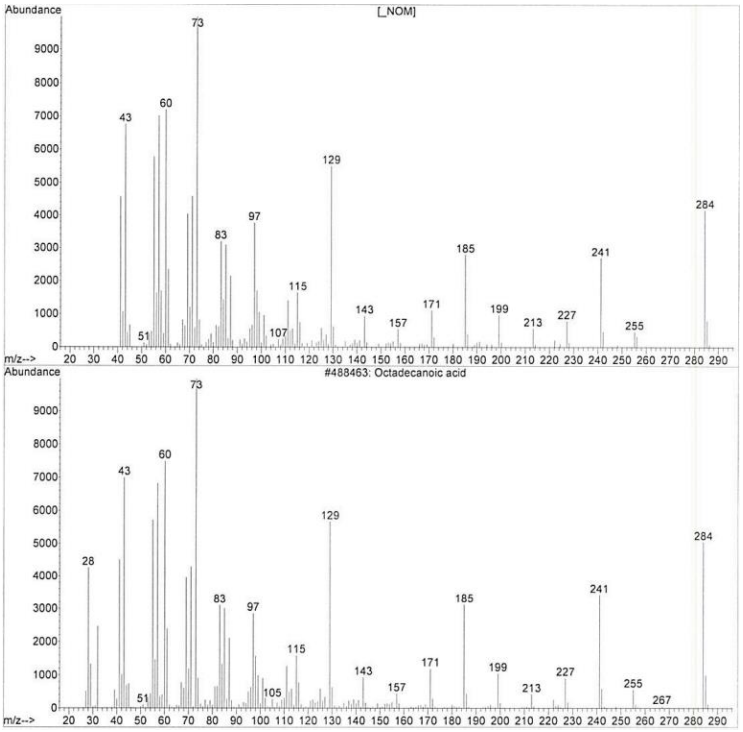

Figure A13 Mass spectrum of octadecanoic acid in N23 using the drug method. This accounts for the peak at 5.715 minutes. The bottom spectrum shows the library match.

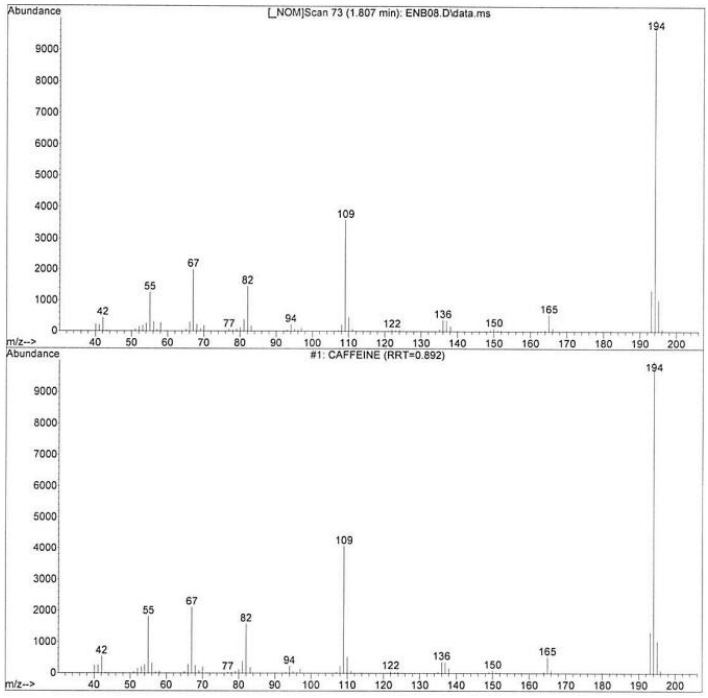

Figure A14 Mass spectrum of caffeine in N24 using the nitazene method. This accounts for the peaks from 1.807 minutes to 1.981 minutes. The bottom spectrum shows the library match.

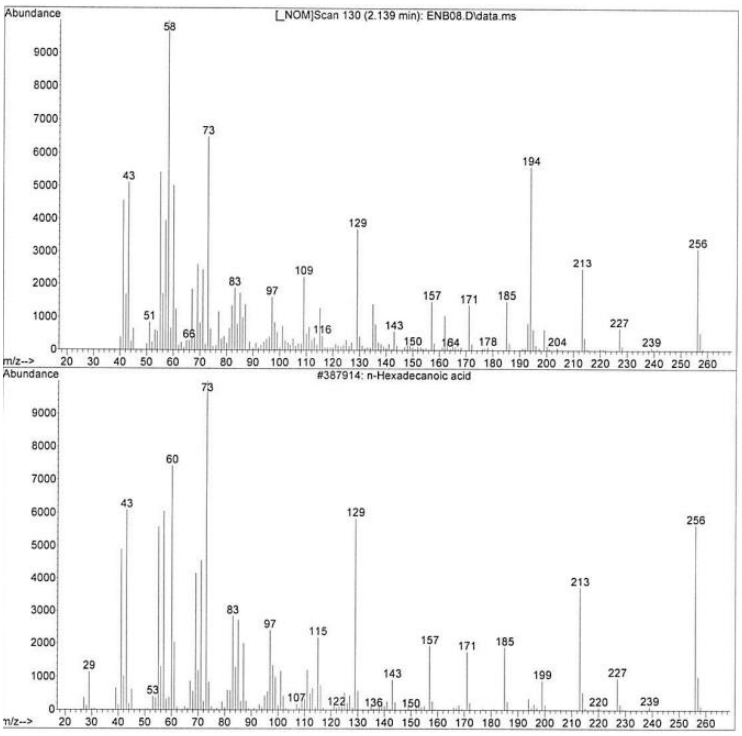

Figure A15 Mass spectrum of n-Hexadecanoic acid in N24 using the nitazene method. This accounts for the peak at 2.139 minutes. The bottom spectrum shows the library match.

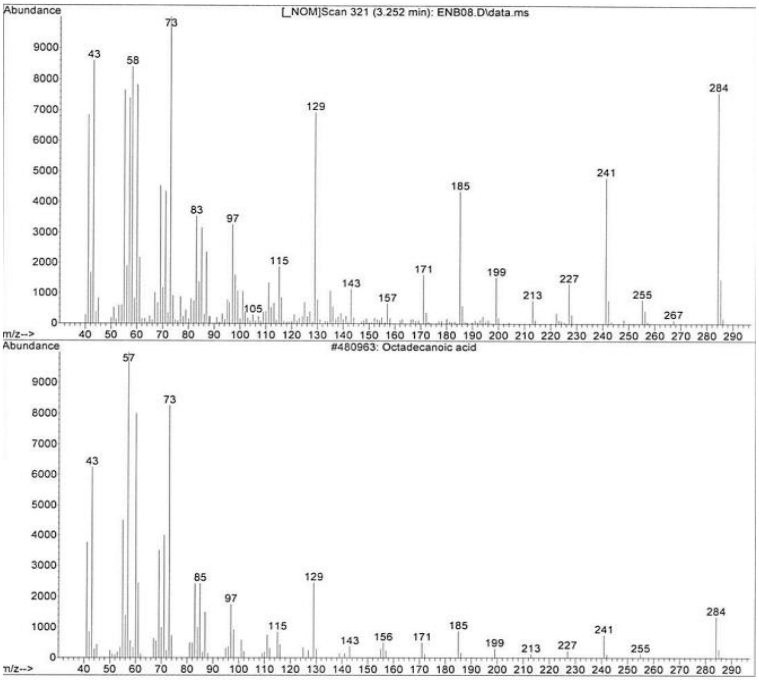

Figure A16 Mass spectrum of octadecanoic acid in N24 using the nitazene method. This accounts for the peak at 3.252 minutes. The bottom spectrum shows the library match.

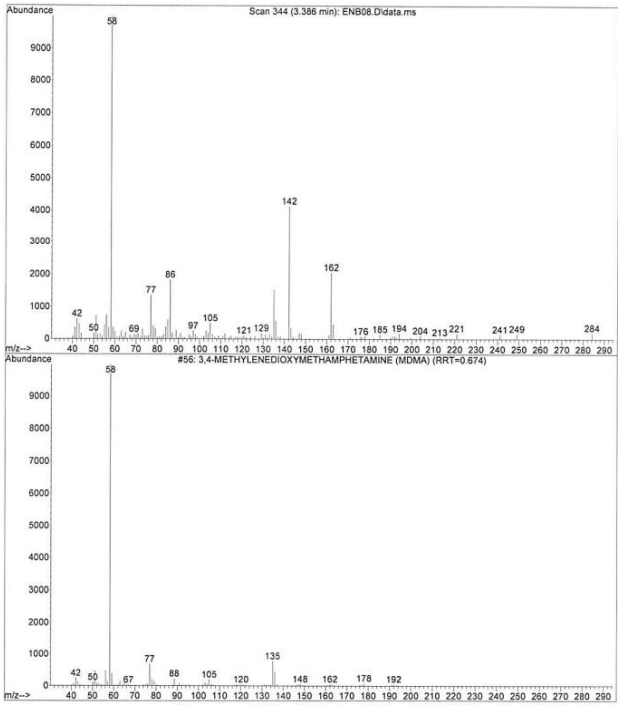

Figure A17 Mass spectrum of MDMA in N24 using the nitazene method. This accounts for the peak at 3.386 minutes. The bottom spectrum shows the library match.

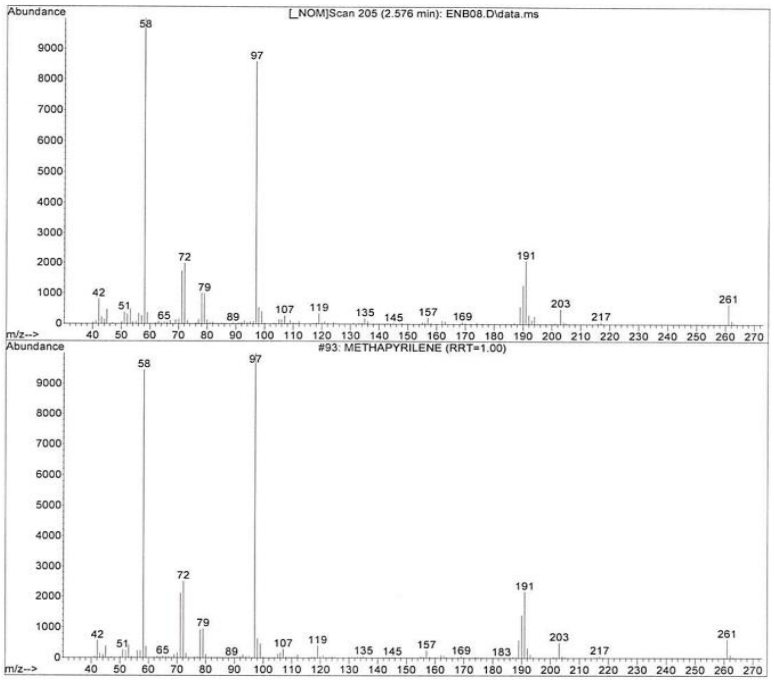

Figure A18 Mass spectrum of internal standard in N24 using the nitazene method. This accounts for the peak at 2.576 minutes. The bottom spectrum shows the library match.

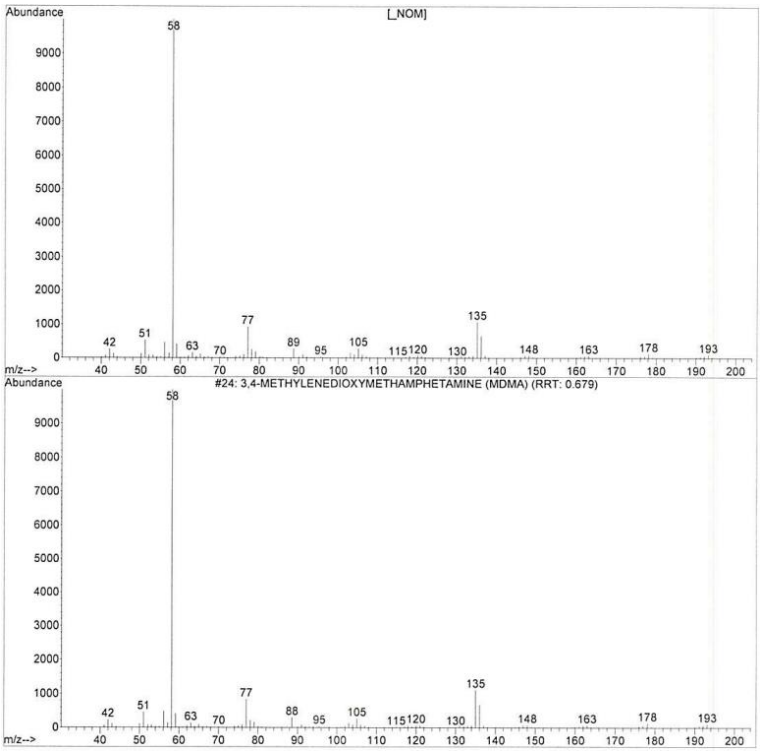

Figure A19 Mass spectrum of MDMA in N24 using the drug method. This accounts for the peaks from 3.577 minutes to 4.539 minutes. The bottom spectrum is the library match.

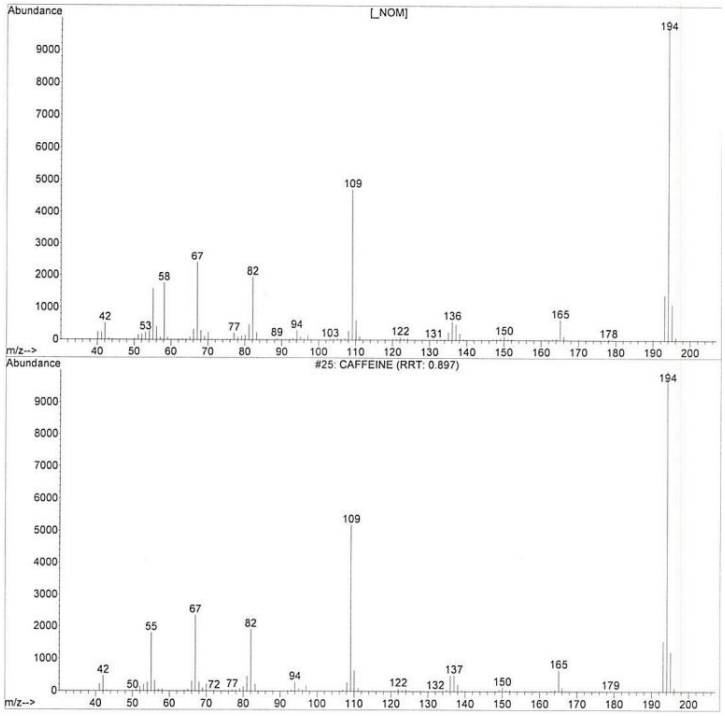

Figure A20 Mass spectrum of caffeine in N24 using the drug method. This accounts for the peak at 4.825 minutes. The bottom spectrum is the library match.

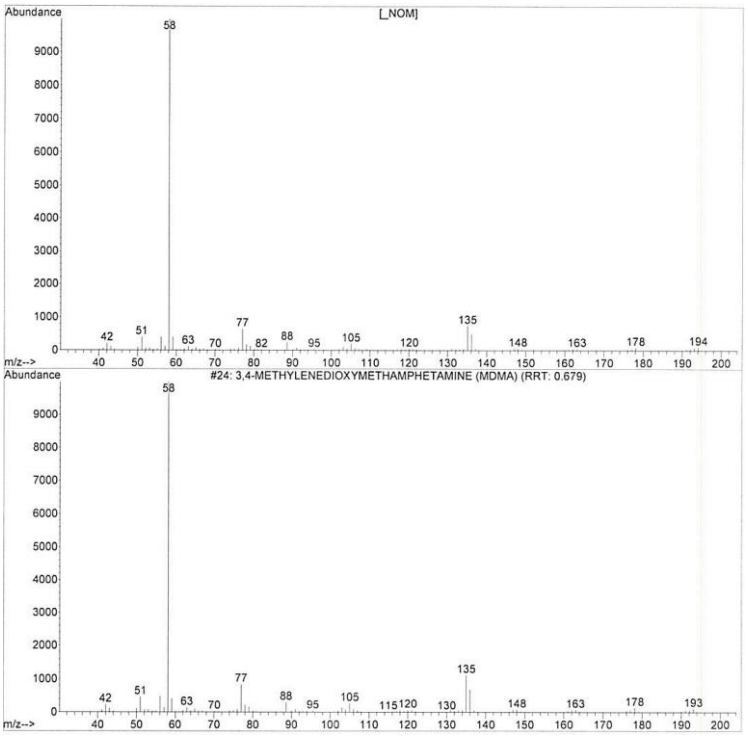

Figure A21 Mass spectrum of MDMA in N24 using the drig method. This accounts for the peak at 4.916 minutes. The bottom spectrum is the library match.

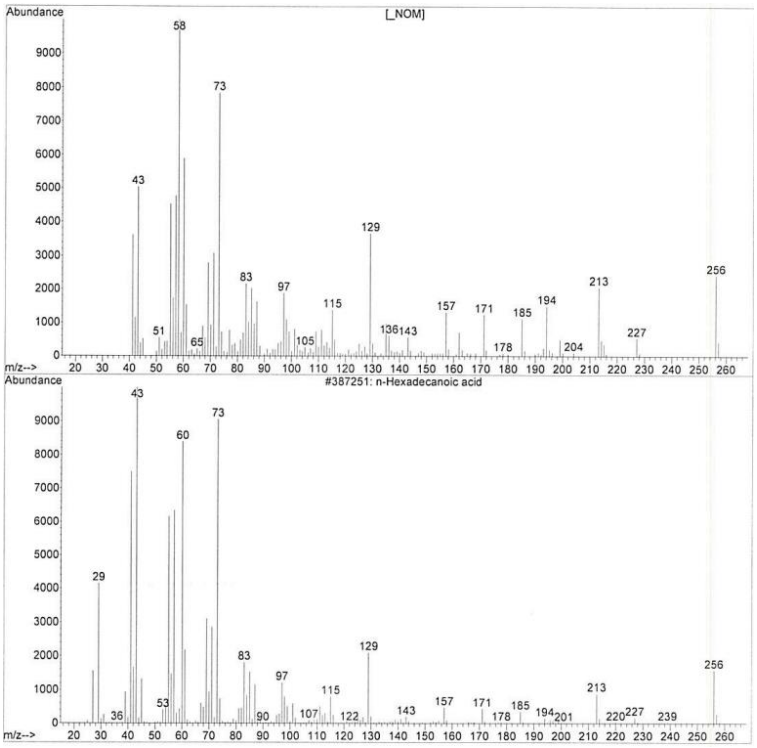

Figure A22 Mass spectrum of n-Hexadecanoic acid in N24 using the drug method. This accounts for the peak at 5.082 minutes. The bottom spectrum is the library match.

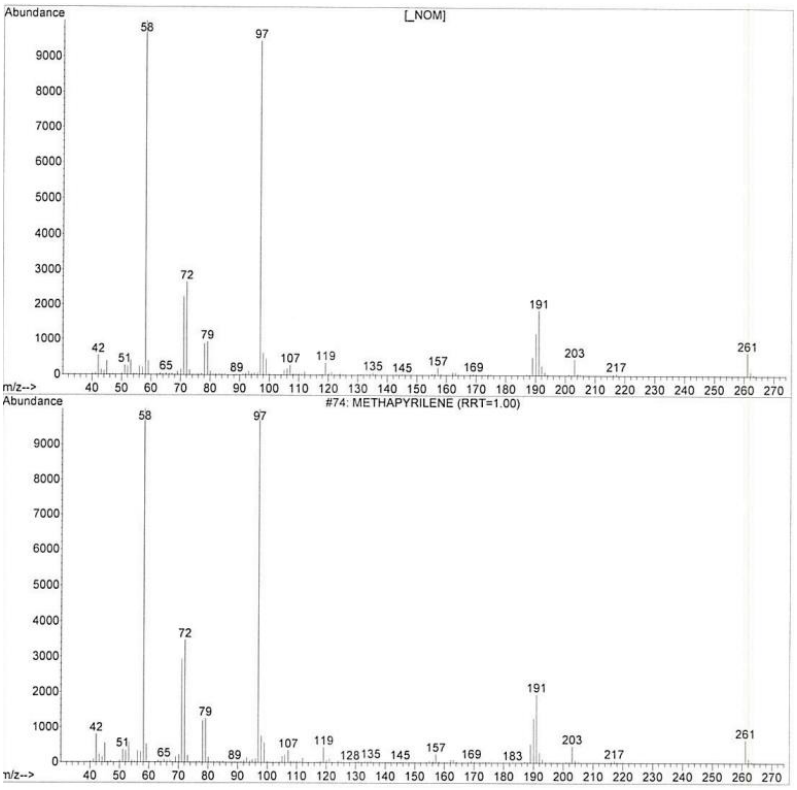

Figure A23 Mass spectrum of internal standard in N24 using the drug method. This accounts for the peak at 5.368 minutes. The bottom spectrum is the library match.

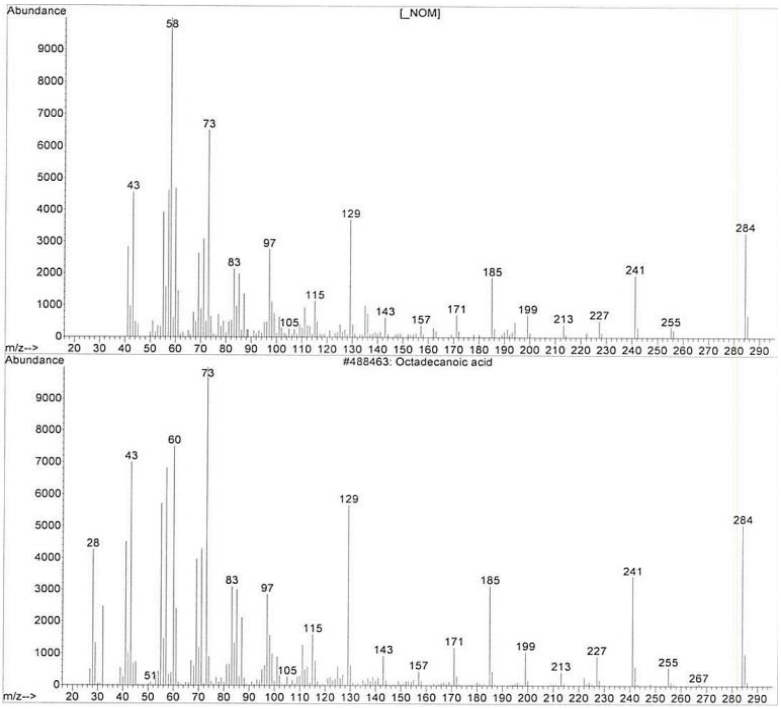

Figure A24 Mass spectrum of octadecanoic acid in N24 using the drug method. This accounts for the peak at 5.716 minutes. The bottom spectrum is the library match.

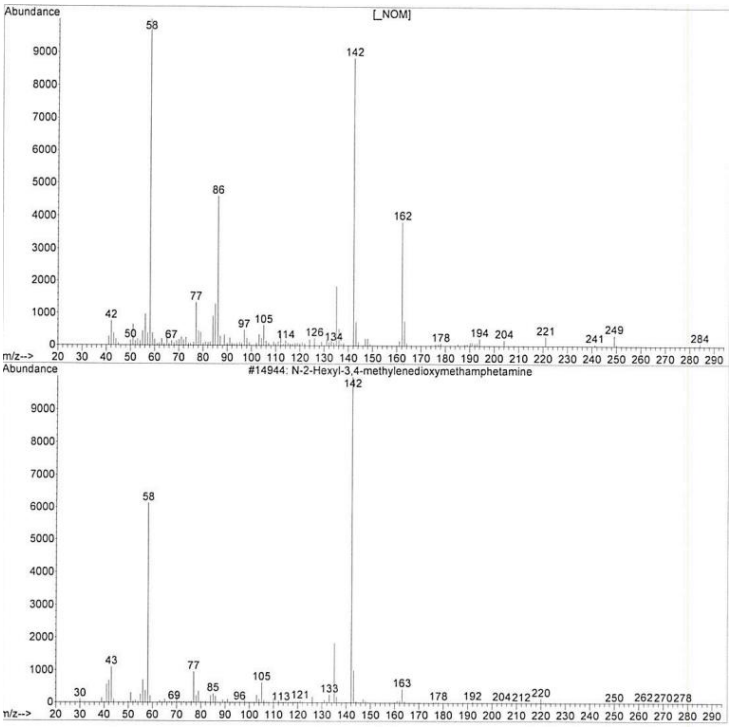

Figure A25 Mass spectrum of N-2-Hexyl-3,4-methylenedioxymethamphetamine in N24 using the drug method. This accounts for the peak at 5.811 minutes. The bottom spectrum is the library match.

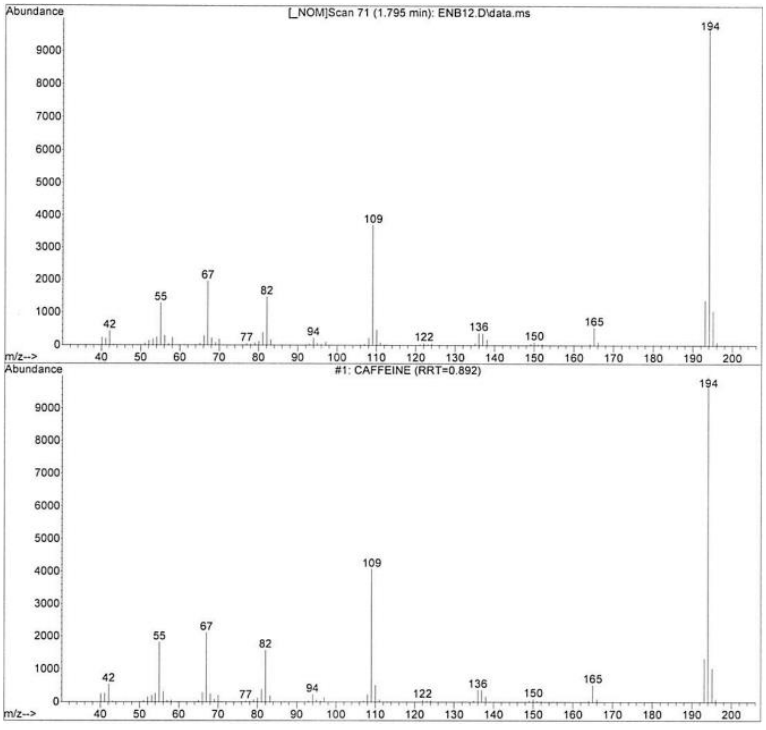

Figure A26 Mass spectrum of caffeine in N25 using the nitazene method. This accounts for the peaks from 1.795 minutes to 2.133 minutes. The bottom spectrum is the library match.

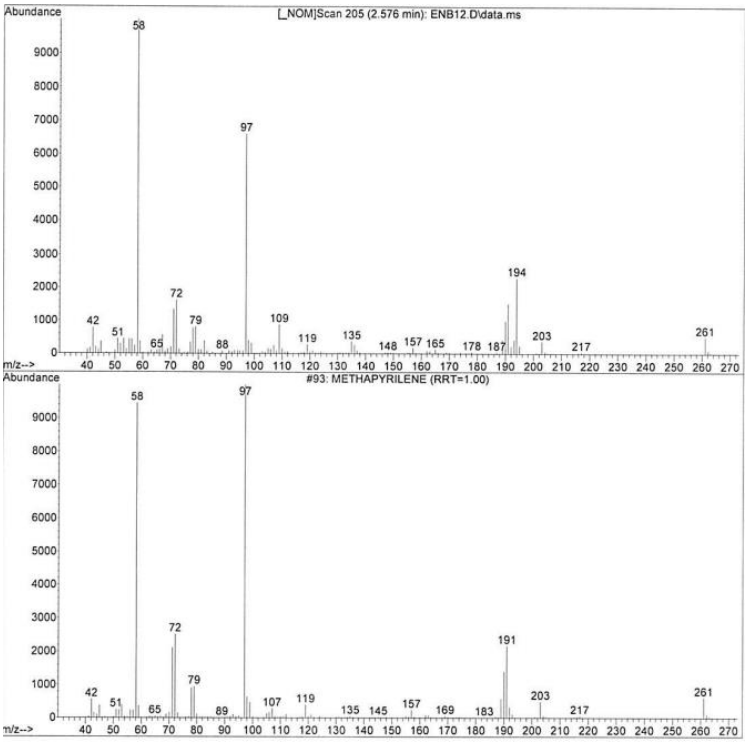

Figure A27 Mass spectrum of the internal standard in N25 using the nitazene method. This accounts for the peak at 2.576 minutes. The bottom spectrum is the library match.

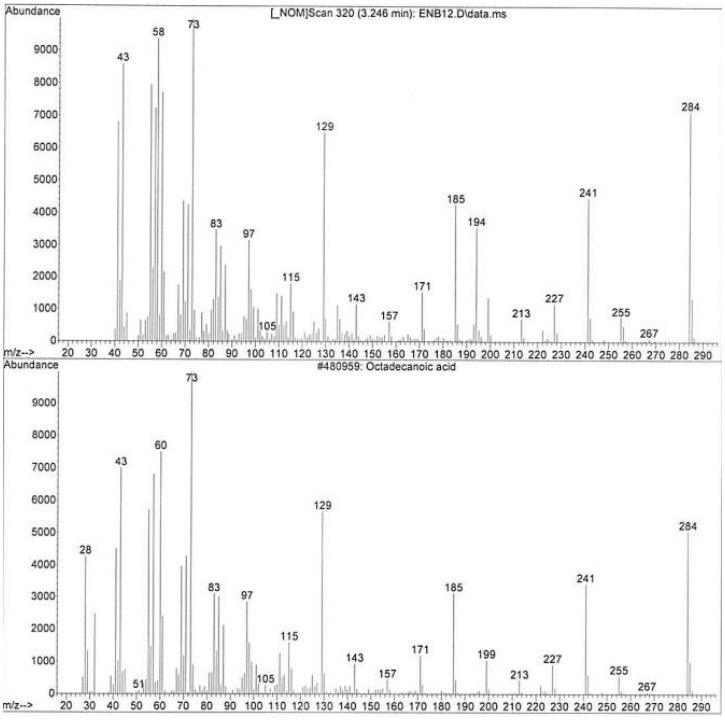

Figure A28 Mass spectrum of octadecanoic acid in N25 using the nitazene method. This accounts for the peak at 3.246 minutes. The bottom spectrum is the library match.

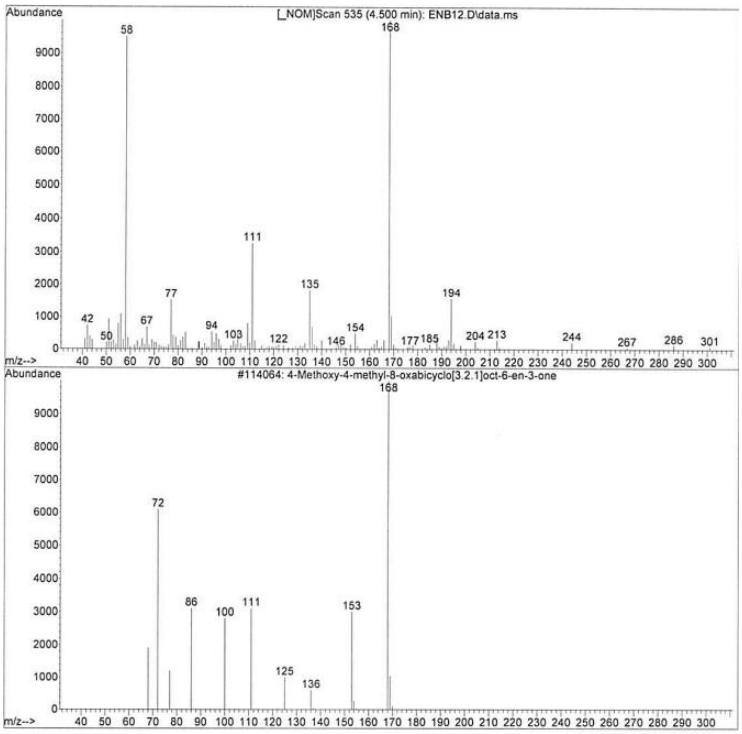

Figure A29 Mass spectrum of 4-methoxy-4-methyl-8-oxibicyclo[3.2.1]oct-6-en-3-one in N25 using the nitazene method. This accounts for the peak at 4.5 minutes. The bottom spectrum is the library match.

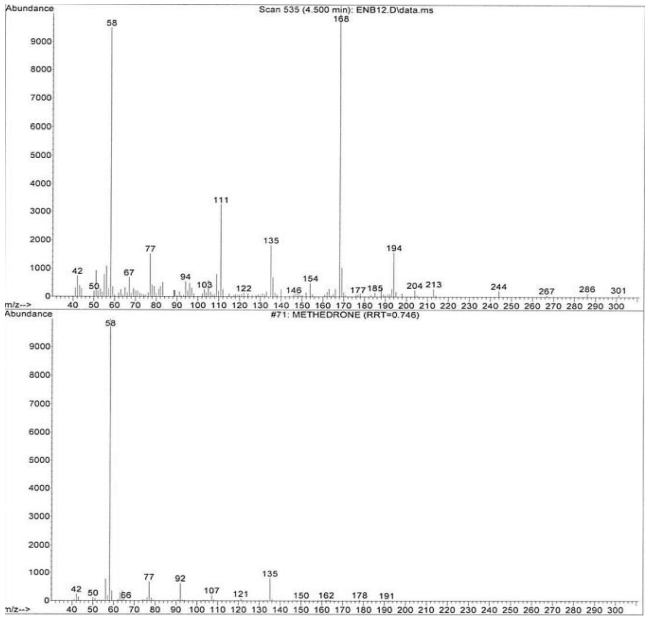

Figure A30 Mass spectrum of methedrone in N25 using the nitazene method This accounts for the peak at 4.5 minutes. The bottom spectrum is the library match.

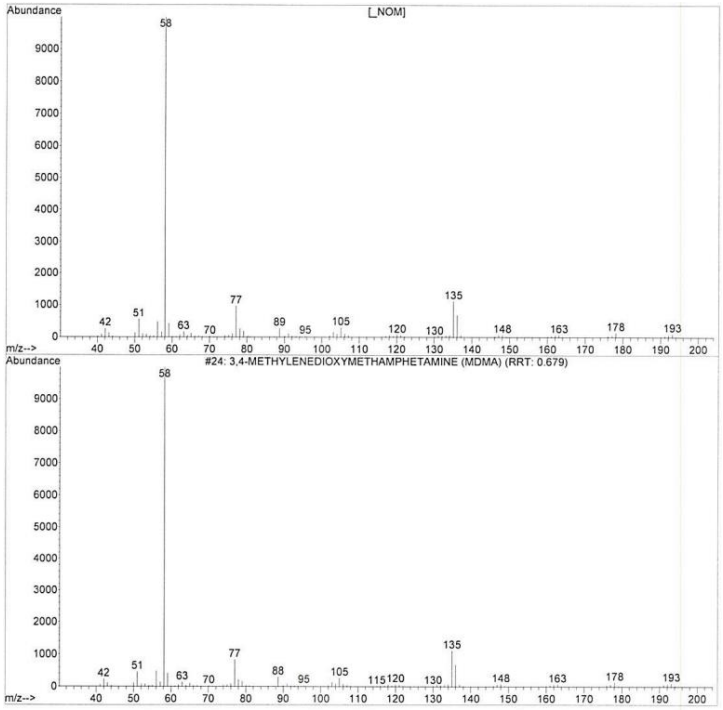

Figure A31 Mass spectrum of MDMA in N25 using the drug method. This accounts for the peaks from 4.682 minutes to 3.682 minutes. The bottom spectrum is the library match.

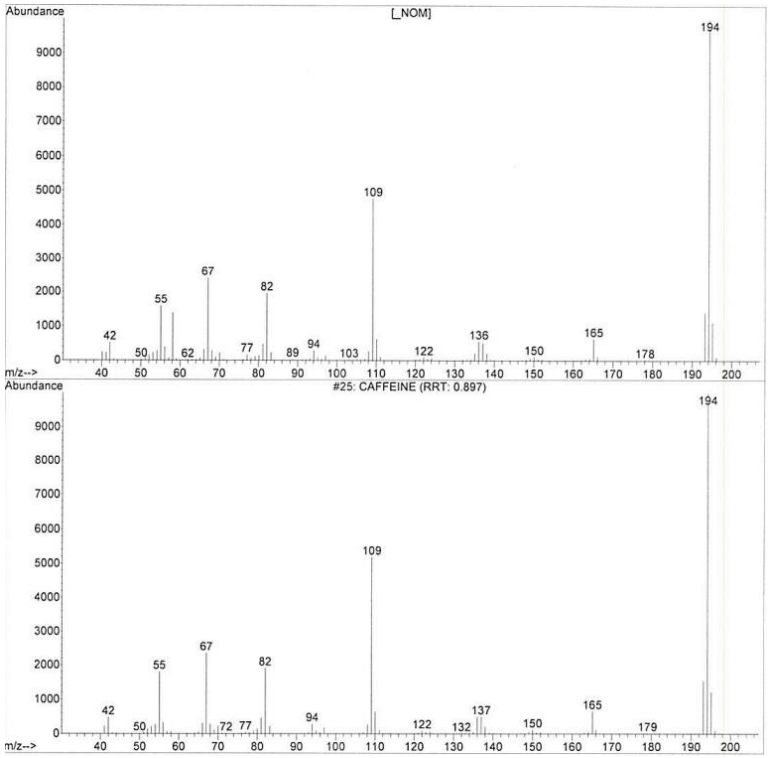

Figure A32 Mass spectrum of caffeine in N25 using the drug method. This accounts for the peak at 4.839 minutes. The bottom spectrum is the library match.

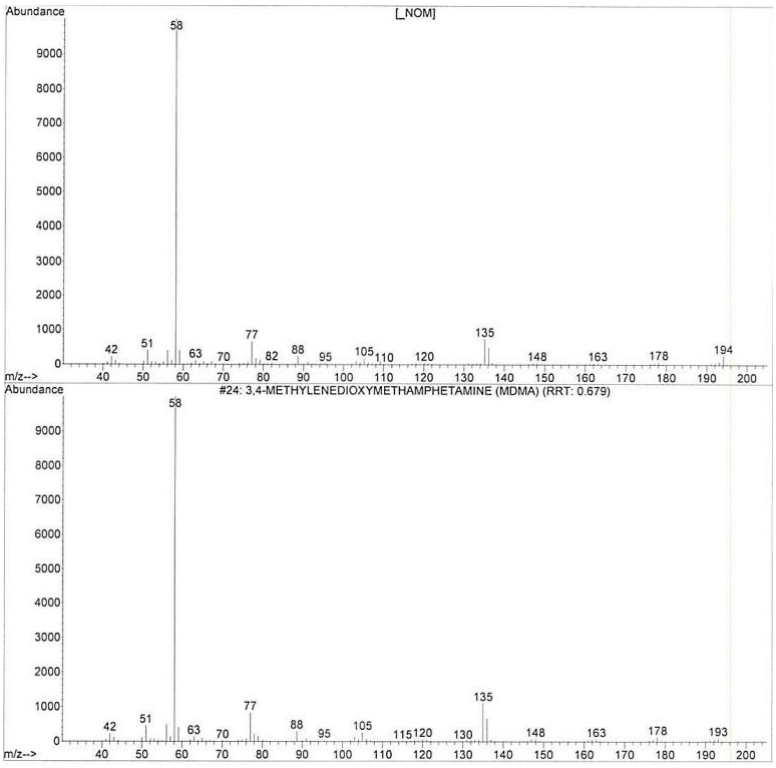

Figure A33 Mass spectrum of MDMA in N25 using the drug method. This accounts for the peak at 4.901 minutes. The bottom spectrum is library match.

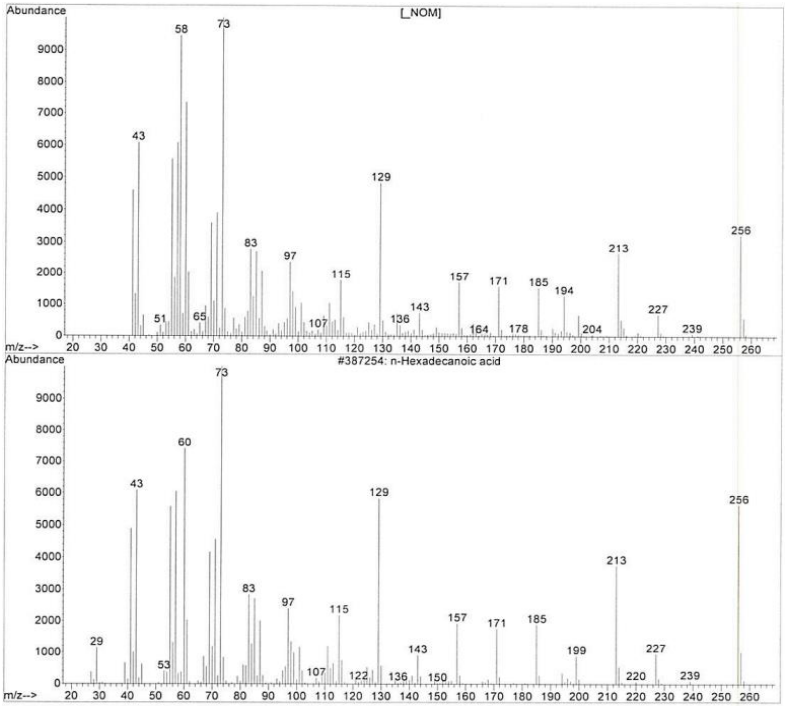

Figure A34 Mass spectrum of n-Hexadecanoic acid in N25 using the drug method. This accounts for the peak at 5.087 minutes. The bottom spectrum is the library match.

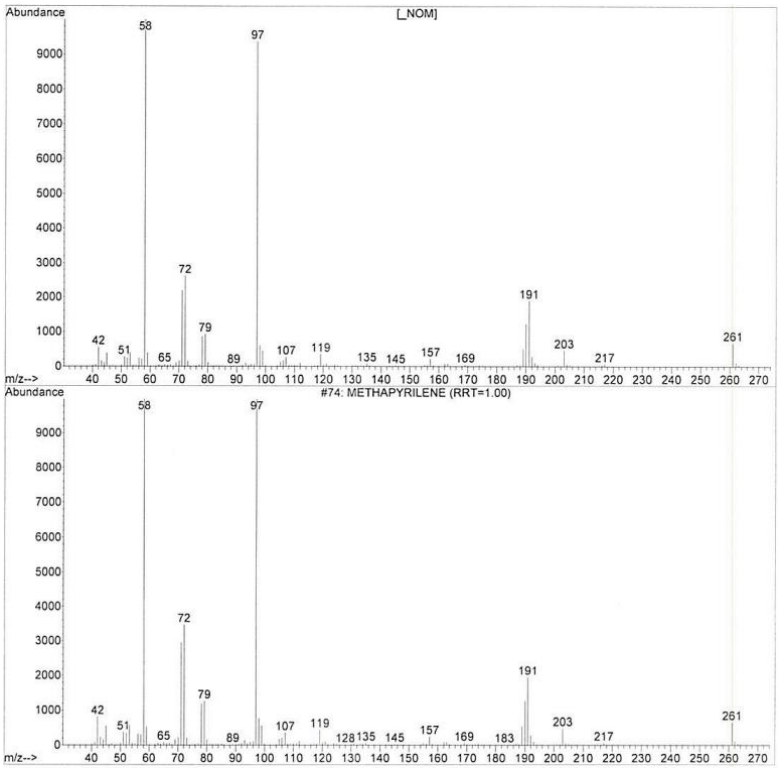

Figure A35 Mass spectrum of internal standard in N25 using the drug method. This accounts for the peak at 5.363 minutes. The bottom spectrum is the library match.

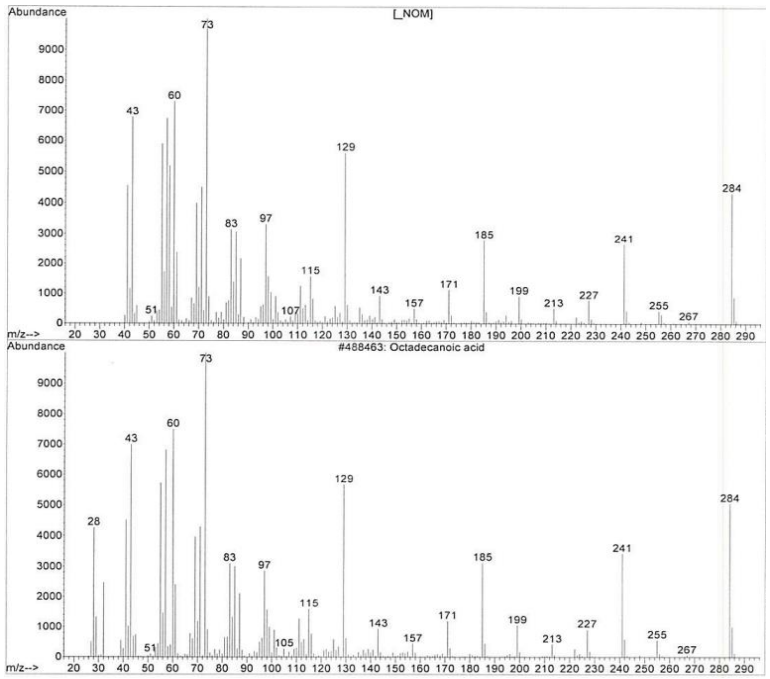

Figure A36 Mass spectrum of octadecanoic acid in N25 using the drug method. This accounts for the peak at 5.715 minutes. The bottom spectrum is the library match.

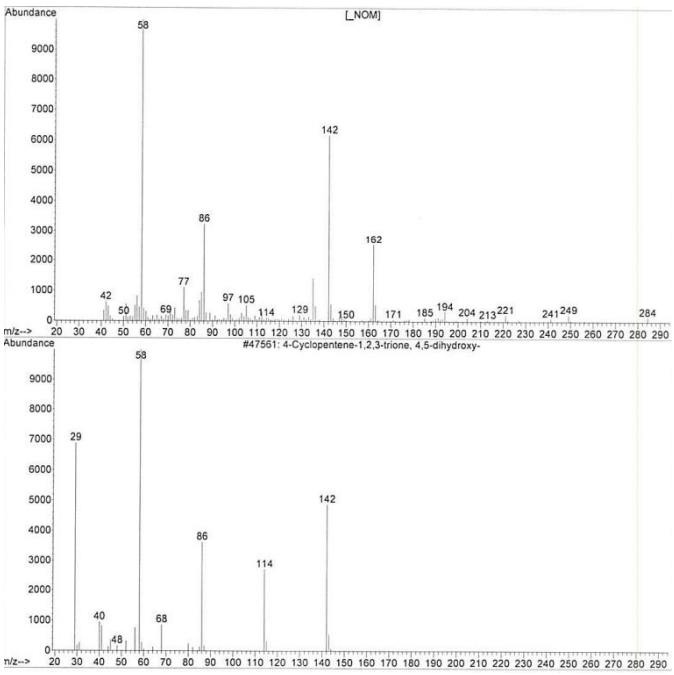

Figure A37 Mass spectrum of 4-cyclopentene-1,2,3-trione, 4,5-dihydroxy- in N25 using the drug method. This accounts for the peak at 5.811 minutes. The bottom spectrum is the library match.

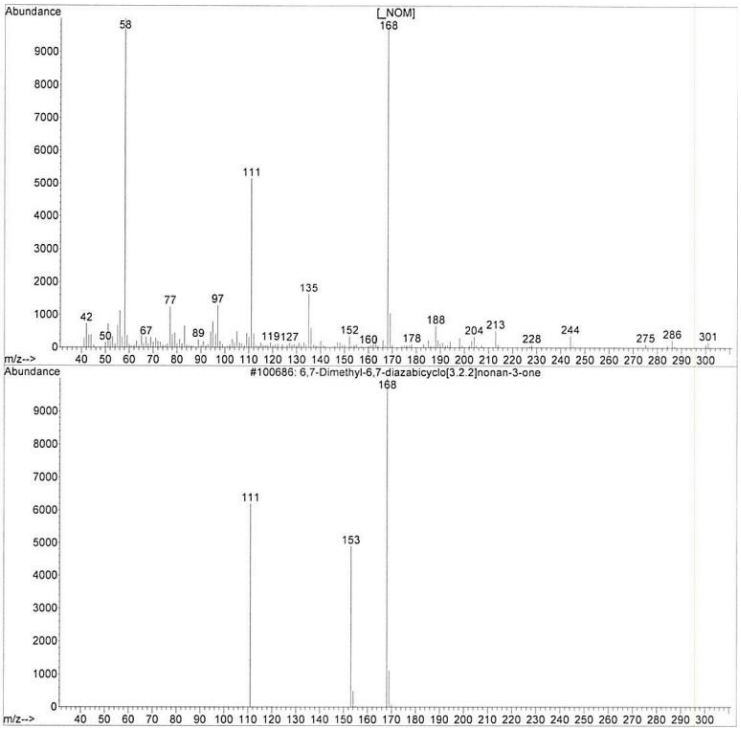

Figure A38 Mass spectrum of 6,7-dimethyl-diazabucyclo[3.2.1]nonan-3-one in N25 using the drug method. This accounts for the peak at 6.463 minutes. The bottom spectrum is the library match.

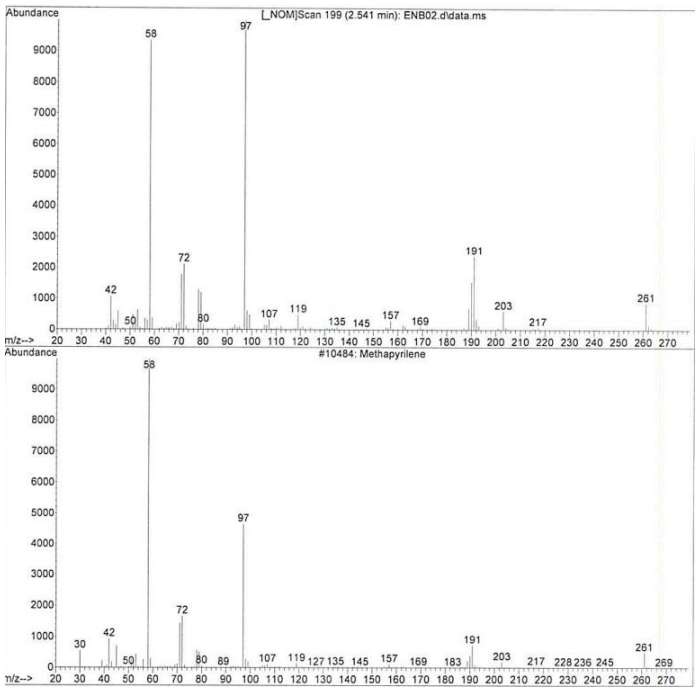

Figure A39 Mass spectrum of internal standard in F13 using the nitazene method. This accounts for the peak at 2.541 minutes. The bottom spectrum is the library match.

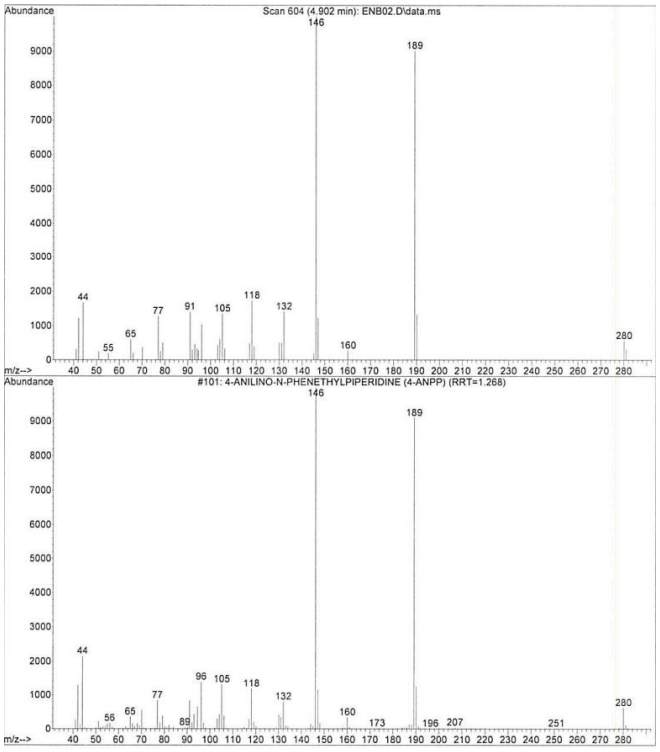

Figure A40 Mass spectrum of 4-ANPP in F13 using the nitazene method. This accounts for the peak at 4.902 minutes. The bottom spectrum is the library match.

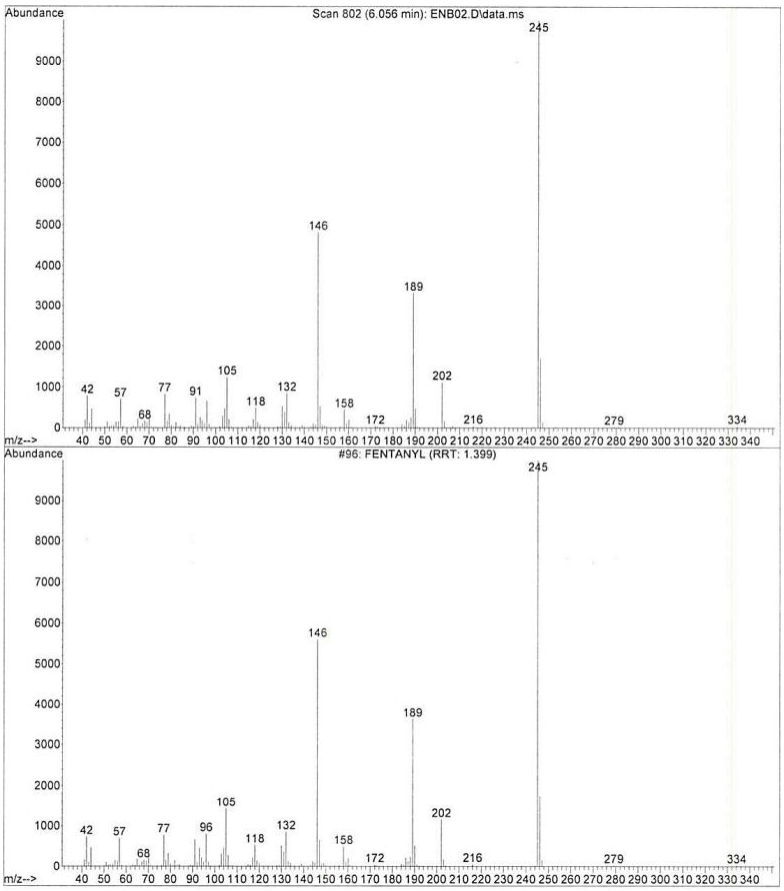

Figure A41 Mass spectrum of fentanyl in F13 using the nitazene method. This accounts for the peak at 6.056 minutes. The bottom spectrum is the library match.