Development of an Untargeted Gas Chromatography-Mass Spectrometry (GC/MS) Method for the Detection of Drugs in Wastewater

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Abstract

Monitoring current illicit drug trends and consumption rates of pharmaceuticals using a non-invasive collection technique is needed to address the present drug use and the growing drug epidemic. Reliance on self-reporting drug use surveys is not always a practical measure of illicit drug use. Wastewater analysis has been used globally as a targeted method for monitoring the consumption of specific illicit drugs. Current existing analytical techniques for wastewater analysis focus on the use of targeted liquid chromatography-mass spectrometry (LC-MS) based techniques. Few gas chromatography (GC) procedures exist for wastewater analysis, and those that do concentrate their methods on a single class of drugs operating their mass spectrometer (MS) in selective ion monitoring (SIM) mode. This study aims to develop an untargeted, underivatized, full scan gas chromatography-mass spectrometry (GC/MS) method for the analysis of wastewater. Solid phase extraction (SPE) was performed with UCT mixed mode, 15 mL Clean Screen DAU columns with 500 mg sorbent to extract a 500 mL wastewater sample. Sample extracts were reconstituted in ethyl acetate and analyzed on a Shimadzu GCMS-QP2020 gas chromatograph-mass spectrometer (GC/MS) installed with an Agilent J&W HP-5MS (30 m × 0.25mm, 0.25 µm) column. Injection volume, flow rate, oven temperature, and ion source scan rate were optimized to develop an untargeted full scan method for the detection of pharmaceuticals. Calibration curves were developed for 42 targeted drugs. A 1 µL sample volume is run with a splitless injection utilizing helium as the carrier gas and the instrument operated in constant flow mode at a rate of 1.0 mL/min. The GC oven program is held at the initial temperature of 70°C for 2 min then ramped at 15°C/min to 300°C and held for 10 min for a total run time of 27.33 min. The injection port, transfer line, and source were set at 250°C, 280°C, and 200°C respectively. The MS operated in full scan mode, scanning ions from 35-550 m/z at an event time of 0.20 seconds from 3.50-27.33 minutes. Out of a 42 drug panel, over 75% of the generated calibration curves were suitable for quantitation with coefficients of determination greater than 0.9875. Limits of detection for most drugs ranged from 0.10-1.0 ng/mL, on par with many targeted liquid chromatography methods. The optimized untargeted method is able to detect a wide range of compounds in addition to those in the drug panel. The untargeted full scan MS method supports monitoring a wider range of pharmaceuticals overlooked in traditional targeted waste water methods such as changing trends in novel psychoactive substances.

Introduction

The Substance Abuse and Mental Health Services Administration's (SAMHSA) 2018survey results show an increase in the use of nearly every drug class across all surveyed age groups (SAMHSA, 2019). Almost 8%, or 19.3 million people, of the United States adult population ($\geq$18), suffer from a substance use disorder (SUD), and nearly 40% of their SUDs are struggling with illicit drugs (SAMHSA, 2019). Since the early 1990's, over prescribing of pain management drugs has led the United States into the middle of an opioid epidemic. It is estimated that between 21-29% of patients prescribed opioids will misuse them (Vowles et al., 2015). Even more alarming is in the period of one year, 2016-2017, more populous cities experienced a 54% increase in opioid overdoses, and a 70% increase in midwestern states (Vivolo-Kantor et al., 2018). However, while developed strategies to combat this epidemic have been successful in reducing illicit heroin use by over 2 million users from 2017-2018, prescription opioids, such as methadone and buprenorphine, abuse has continued to rise (SAMHSA, 2019). Additionally, marijuana, stimulant, and hallucinogenic drug use has significantly (statistically significant at the 0.05 level) increased in use by the $\geq$26 age population over this same time frame (SAMHSA, 2019).

Over the last ten years the Drug Enforcement Administration (DEA) has reported an 88% decrease in domestic laboratory production of methamphetamine, yet during this time high purity ($\geq$95%) Mexican border drug seizures have increased nearly tenfold, and overdose deaths involving methamphetamine have increased by 500%. Overdose (OD) deaths involving most illicit substances have continued to rise steadily since 1999, but nearly a 30% decrease from 2015-2019 in OD deaths involving solely heroin has been reported (DEA, 2019). Prescription pharmaceutical OD deaths also continue to rise. From 1999-2017, OD deaths involving

prescription antidepressants, psychostimulants, benzodiazepines, and opioids have risen 100-300%. Scarier yet, OD deaths involving fentanyl or fentanyl analogs have increased over 1000% in the last five years (DEA, 2019).

The American Addiction Centers (AAC) report that current admissions to drug treatment programs have risen over the last three years while government funding for these programs has decreased. Almost 65% of treatment facilities received government funding in 2002 but since then this has decreased steadily leading to less than 50% of treatment centers receiving government funding today (AAC 2019). This has resulted in a decrease in non-profit free treatment programs and a 21% increase in for-profit treatment programs since 2004 (AAC 2019). In the latest data from 2017, the Center of Disease Control and Prevention (CDC), reported a 9.6% increase in deaths attributed to overdose in the United States at over 70,000 deaths. Utilizing Healthcare Cost and Utilization Project's (HCUP) Nationwide Emergency Department Samples (NEDS) the CDC generated data accounting for nearly one million non-fatal OD cases treated by emergency departments in 2017 alone (Vivolo-Kantor et al. 2017). In 2008, SAMHSA stated that for prescription painkillers alone, for every overdose death there are over 30 non-fatal overdoses and over 800 non-medical abusers (SAMHSA, 2010). Given the increase in drug consumption and overdose cases, a comprehensive, non-invasive method for monitoring community drug use is necessary.

Current monitoring techniques as practiced by agencies like SAMHSA, the DEA, the CDC are no longer an effective or accurate way of monitoring real time drug trends. Self-reporting population surveys monitoring drug use is an economical choice that has significant limitations. Self-reporting can lead to bias, misrepresentation, and underreporting of the true frequency of drug use. Misrepresentation occurs due to the negative stigma surrounding the use

of illicit substances and abusing prescription pharmaceuticals. Additionally, in many instances, illicit drug users do not know exactly what drugs they have been using, reporting solely heroin use when in actuality they have been using fentanyl, a fentanyl analog, or some polydrug mixture. Therefore, surveys are often ignored by these users or falsified and the information given is not an accurate representation of the true value. Self-reporting also lends itself to being open to population bias and is dependent on the area surveyed to be an accurate representation of a larger population. Issues arise when urban areas are overly represented in these surveys leading to the belief that illicit drug use is solely a problem for larger cities (Banta-Green et al., 2009; van Nuijs et al., 2009b). Lastly, the greatest issue with survey-based data is the rate at which it becomes available. Often, surveys are done on a biennial basis, with resulting data processed and released the following year. Thus, by the time data is made available it is nearly three years old and no longer an accurate representation of the current state of affairs.

Similarly, using overdose statistics put out by the CDC lends itself to some of the same issues as surveys. Although it avoids the bias that occurs with self-reporting, the data is collected over an extended period of time and not published or made available for over a year after collection. In many instances, hospitals are not required to report overdoses and, oftentimes, treat the symptoms of an overdose without knowing the underlying cause leading to the possibility of misreported, or underreported, information. In cases resulting in death, accidental overdose information is often reported as an unspecified overdose. Over a six year study in Indiana, it was shown that over 57% of overdose death cases were attributed as unspecified overdoses (Lowder et al. 2018). Overdose statistics also do not account for overdoses that occur and are treated at home. Naloxone can be prescribed as a treatment for an opioid overdose, but does not necessarily mean it is needed or used. Arizona, California, Ohio,

Rhode Island, Vermont, and Virginia all require physicians to co-prescribe, or offer to co-prescribe, naloxone in situations where prescribed opioid medication exceeds a certain dosage. Therefore, monitoring prescriptions is also not an accurate way of monitoring drug use.

Using arrest records for monitoring drug use can also lead to an inaccurate representation of population. The Federal Bureau of Investigation (FBI) compiles annual arrest records based on a multitude of charges, of which drug offenses is one category. The Uniform Crime Reporting (UCR) statistics are an excellent tool for identifying isolated drug use but only accounts for those arrested, not the entire scope of communal drug use. Monitoring drugs based solely on arrest does not account for users who are never arrested and also includes repeat offenders multiple times. Reported cases encompass both sales/distribution as well as possession charges. Misdemeanor possession charges have risen every year for the last ten years (FBI 2019). Simple possession charges are sometimes reported as "possession of a controlled substance" where the substance is either unidentified or categorized as "other," making the information unreliable in identifying specific drug trends. The National Forensic Laboratory Information System (NFLIS) aids this by keeping records of the number of cases, amounts, and types of drugs submitted. However, not all crime labs report their case statistics to NFLIS, leading to a population bias. Arrest monitoring also suffers from the same delay as other long-term analysis in that information is not made available till a year later. Additionally, the 156 participating drug laboratories reported having nearly 300,000 cases in backlog, meaning stats obtained from the current years report does not fully reflect current drug trends (DEA 2019).

A more comprehensive method for the accurate monitoring of drug use could be the testing of municipal wastewater. Analyzing water sources for the presence of drugs and other compounds is hardly a new concept with extraction and instrumental techniques dating back to

more than fifty years ago. Many early methods were developed for the detection of pesticides in natural waterways (Abbott et al., 1967; Hindin et al., 1962; Leoni, 1971; Teasley et al., 1963). Adapting these methods for testing wastewater for pharmaceutical consumption was first theorized in 2001 by Environmental Protection Agency (EPA) scientist Christopher Daughton (Daughton, 2001). According to the Organization for Economic Co-operation and Development (OCED) more than 75% of the United States population has plumbing that is serviced by a wastewater treatment plant (WWTP), with this percentage rising dramatically in more urban settings. Each WWTP services a designated area and therefore measuring concentrations of influent wastewater provides a more accurate representation of localized drug consumption. Samples from WWTP can be collected in bulk and enable real time tracking of drug trends on a more frequent basis. Some WWTP can be reluctant to provide samples, or allow individuals to collect them, citing possible ethical or privacy issues. Though this may have some merit in regards to sampling of smaller populations such as manholes, schools, or individual sewer lines, utilizing wastewater to monitor large populations does not pose any ethical concerns as individualization is impossible (Hall et al., 2012). Additionally, not unlike garbage or solid waste, once an individual flush their bodily excretions it can be considered discarded and privacy is no longer expected, therefore can be collected without obtaining a warrant (Hering, 2009). However, drug levels in wastewater can be contributed by more than just drug consumption and excretion (Figure 1). In addition to user excretions, pharmaceutical disposal as well as clandestine laboratory waste may contribute to elevated levels in certain WWTPs, however, it is suspected that this contribution would be minor compared to user contributions.

The primary goal of WWTPs is to render wastewater safe for environmental reintroduction. Treatment consists of a six-step process: screening, primary clarification,

biological processing, secondary clarification, filtration, and disinfection. WWTPs may use all or some of these techniques (Rao et al., 2013). Raw incoming wastewater, or influent, is initially screened and filtered utilizing mesh or bar screens to remove large solid material. Heavy particles of sand or grit are also removed by vortexing, forcing these particles to the bottom. Primary clarification removes suspended semisolids, or sludge, from the wastewater by simple sedimentation. Remaining sludge particulate and dissolved organic solids are removed by biological processing in which the water is treated by aeration and the introduction of microorganisms that digest these solids. The introduction of these microbes results in cellular flocs which require removal via a secondary clarification step. Wastewater is then filtered, typically using a simple carbon filter, and sterilized using ultraviolet radiation, ozonation, or chemically by chlorination/dichlorination. Post treated wastewater, or effluent, is then reintroduced into the environment by dumping into natural waterways or used for irrigation (Rao et al., 2013; Stephens et al., 2009).

The use of wastewater for illicit substance monitoring has been performed globally; including New Zealand, Australia, China, South Africa, many countries in Europe, as well as the United States, for over the last twenty years, employing a variety of extraction and instrumental analysis techniques. In 2011, a critical review was published evaluating many of these analytical techniques and their results focusing on cocaine, amphetamine, cannabinoids, and opiates (Van Nuijs et al., 2011). A majority of these techniques employ the use of a solid phase extraction (SPE). Solid phase extractions can be modified both in the sorbent material as well as the extraction scheme used. This allows for extremely clean and compound specific extractions. SPE cartridges are commercially available with class specific sorbent materials. In Van Nuijs' 2011 review he published a wide variety of different compound specific extractions (Bijlsma et

al., 2009; Boleda et al., 2007; Bones et al., 2007; Castiglioni et al., 2006; Gheorghe et al., 2008; González-Mariño et al., 2009; Huerta-Fontela et al., 20017; Hummel et al., 2006; Kasprzyk-Hordern et al., 2008; Mari et al., 2009; Postigo et al., 2008a; van Nuijs et al., 2009a; van Nuijs et al., 2011; Zuccato et al., 2005), columns used, and extraction schematics. Methods described in these studies used 5-500 milliliters (mL) of sample, depending on the sample type and analysis being performed. Since 2011 additional SPE methods have been developed (Table 1). Fewer extraction methods employ the use of a liquid-liquid extractions (Arbeláez et al., 2014; Baker et al., 2012; Teasley et al., 1963) due large sample and solvent volumes and the greater likelihood of contamination. SPE is typically performed within three days of sample collection, or immediately after samples thaw from frozen to prevent drug metabolism and degradation. Due to particulate matter, samples are better suited for SPE if they have been filtered and/or centrifuged prior to extraction. In rarer instances no extractions were performed and raw wastewater samples were injected directly onto the instrument for analysis (Chiaia et al., 2008).

Historically, analysis of extracted samples are commonly analyzed by either high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC) coupled to a mass spectrometer (MS). Numerous LC-MS methods have been developed using various columns and solvent systems based on the analytes of interest (Table 2). Rarely are these methods developed as a full scan but instead are developed in specific drug panels based on class of drug. Therefore, these methods are operated in one of two modes, selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) solely focusing on specific ions for a select group of analytes. Modern LC systems have the advantage of being extremely sensitive and allow for greater injection volumes, thus, require a less concentrated injection resulting in reduced sample volumes.

Gas chromatography (GC) mass spectrometry (MS) is a less common analytical technique utilized for the analysis of wastewater. Fewer GC methods exist compared to their LC counterparts possibly due to their decreased sensitivity. GC requires far less startup cost than LC and the utilization of a carrier gas instead of solvent based mobile phases lends it to vastly cheaper maintenance, operation, and waste disposal fees. Although, some GC methods for the analysis of wastewater have been developed (Table 3). These methods utilize the same sample prep, sample volume, and SPE extraction techniques as LC analysis but rely on an additional derivatization step. The addition of this derivatization has both added time and cost to the wastewater analysis. Furthermore, GC methods are typically performed having a targeted analysis operating in selected ion monitoring (SIM) mode. This is done in an effort to negate background, sample, and atmospheric interferences. SIM mode however narrows the focus of the GC limiting the number of compounds the method can detect, whereas operating in scan mode detects all possible analytes.

The development of an untargeted, underivatized, full scan GC/MS method for the analysis of wastewater is necessary for the accurate monitoring of communal drug use. This will allow for the detection of a wide array of pharmacologically active compounds, including prescription medicines, over-the-counter therapies, and illicit drugs. A method for quantitating 42 common drugs will also be developed.

Materials & Methods

Sample Collection

Wastewater samples were collected from nine partnering WWTPs throughout central and eastern Virginia. Both influent and effluent samples were collected from each WWTP as well as a sample from natural waterways where samples were reintroduced to the environment. A

minimum of 500 mL of each sample (influent, effluent, reintroduced) were collected and marked

with the WWTP collection site as well as the date collected. Samples were collected in clean

one-liter plastic bottles, free of preservatives, and stored in the freezer ($\leq$-15°C) until analysis

could be performed. In the cases where multiple bottles of sample were collected one was

placed in the freezer and one was stored in the refrigerator (~4°C) for stability analysis.

Sample Preparation

Prior to extraction 500 mL sample aliquots were allowed to reach room temperature and were

then buffered to pH 6.0 ± 0.10 with the use of buffering salts. Sodium phosphate monobasic

monohydrate and sodium phosphate dibasic heptahydrate were obtained from Fisher Scientific

(Fisher, Hampton, New Hampshire, USA) and used to adjust samples to have a molarity of 0.10

and a pH of 6.0. To each 500 mL sample 6.28 grams of sodium phosphate monobasic

monohydrate and 1.21 grams of sodium phosphate dibasic heptahydrate were added to achieve a

0.1M phosphate buffered solution. Salts were added to each sample and shaken until completely

dissolved. The pH of samples was evaluated on a Thermo Orion Star (Thermo, San Jose,

California, USA) benchtop pH meter calibrated daily using a three-point calibration curve with

pHs of 4, 7, and 10 utilizing calibration buffers from Fisher Scientific. After buffering, if

samples tested pH fell outside the 6.0 ± 0.10 range they were adjusted using 0.1M hydrochloric

acid (HCl) or 0.1M ammonium hydroxide (NH_4OH). The HCl or NH_4OH solutions were added

dropwise to the sample which was shaken vigorously and allowed to settle prior to the pH being

retested. Calibration working solutions and internal standard (IS) working solutions were

prepared from 1.0 mg/mL drug standard (Table 4) certified reference materials purchased from

either Cerilliant (Cerilliant, Round Rock, Texas, USA) or Cayman Chemical (Cayman, Ann

Arbor, Michigan, USA). Calibrators were fortified at concentrations of 0.1, 0.2, 0.4, 1.0, 2.0,

3.0, and 4.0 ng/mL or 50, 100, 200, 500, 1000, 1500, and 2000 ng total for each drug. All

calibrators, samples, and controls were also fortified with 1.0 ng/mL, or 500 ng total, of two

different deuterated internal standards, methadone-d9 and phenobarbital-d5. After being

fortified, buffered samples falling within the proper pH range (5.9-6.1) were then ready for

extraction.

Solid Phase Extraction

The SPE operated using a UCT Positive Pressure Manifold System (United Chemical

Technologies, Bristol, Pennsylvania, USA) connected to an Airgas nitrogen tank (Airgas,

Richmond, Virginia, USA). The SPE extraction was performed using UCT mixed mode, 15 mL

Clean Screen DAU SPE columns with 500 mg sorbent. Extraction solvents; methanol, acetic

acid, ethyl acetate, methylene chloride, isopropanol, and ammonium hydroxide were all obtained

from Fisher Scientific, 98% pure hexanes was obtained from Alfa Aesar (Alfa Aesar, Haverhill,

Massachusetts, USA). SPE columns were conditioned with 3 mL of methanol, 3 mL of ultra-

pure water, followed by 3 mL of 0.10M sodium phosphate buffer pH 6.0. All conditioning steps

were allowed to flow through via gravity. The buffered 500 mL sample was then loaded on

column 10-15 mL at a time and at a flow rate of 10 mL/min. Columns were then washed with 3

mL of ultra-pure water followed by 2 mL of 1.0M acetic acid passed through at 1 mL/min. After

washing columns were then dried at 35 psi for a minimum of 15 minutes (min) with additional

time if columns were not fully dry. Once dry columns were washed with 3 mL hexanes prior to

elution of the acidic fraction with 3 mL of 50:50 (*v:v*) hexane: ethyl acetate, eluted at 1 mL/min

into new properly labeled disposable culture tubes. Columns were then again washed with 3 mL

of methanol at 1 mL/min and dried at 35 psi for a minimum of 15 min with additional time if

columns were not fully dry. The basic fraction was then eluted by gravity into new properly

labeled disposable culture tubes with 3 mL 78:20:2 (*v:v:v*) methylene chloride: isopropanol: ammonium hydroxide. Eluted samples were then evaporated to dryness under a gentle stream of nitrogen with the use of a RapidVap Vertex Drydown Evaporator (Labconco, Kansas City, Missouri, USA). Samples dried at <5 psi at 40°C and checked frequently. As soon as a tube was evaporated to dryness it was removed to prevent blowing away of analytes or thermal degradation. Dried samples were then reconstituted in 100 µL of high purity ethyl acetate and transferred to a 9mm clear screw cap gas chromatograph (GC) autosampler vial with a 200 µL insert. Samples were then ready for instrumental analysis.

Column Capacity

Initial concerns were about how large of a sample volume would be too large and overload the extraction column. Using the standard extraction method, variable sample sizes of 25, 50, 75, and 100 mL were used to determine at what point the column was not binding to additional analyte. One of the wastewater samples known to have a high level of nicotine was used as the sample source. The extracted samples were then run on the GC/MS and nicotine peak abundances were compared.

Solid Phase Extraction Rate

SPE extractions require multiple conditioning, wash, and elution steps in addition to sample introduction. Working with a large sample volume of 500 mL can cause this extraction process to be tedious and lack efficiency. Many SPE methods that utilize a positive pressure manifold recommend allowing the sample to pass through at a rate of 1 mL/min. Conforming to this extraction rate would cause the entire extraction process to be upwards of ten hours prior to

instrumental analysis. A small experiment was designed to evaluate the recovery of two compounds, nicotine and methadone, at various extraction rates. Samples (n=3) were all fortified with 1000 ng total of both analytes and extracted at rates of 1, 5, and 10 mL/min and their resulting recoveries were compared in order to evaluate an optimal extraction flow rate.

Gas Chromatography-Mass Spectrometry Optimization

Samples were to be run on a Shimadzu gas chromatograph-mass spectrometer (GC/MS) model GCMS-QP2020 (Shimadzu, Koyoto, Japan) optimized for the analysis of wastewater. Prior to sample introduction, proper autosampler vials and caps were evaluated. Samples were reconstituted in 100 µL of ethyl acetate, with a low reconstitution volume it was necessary that the 2 mL autosampler vials be equipped with a 200 µL vial insert so the instrument autosampler was able to obtain a proper sampling. Additionally, the autosampler cap was switched from being rubber septum lined to one lined with Teflon. Using ethyl acetate as the reconstitution solvent results in stripping of the rubber septa resulting in major interference with early eluting compounds (Figure 2). Switching to a Teflon lined cap negates this interference allowing for clearly defined peaks (Figure 3).

Various injection volumes of 0.5, 1.0, and 2.0 µL were evaluated to determine what volume would best be suited for this analysis. Since concentrations of drugs in wastewater can often be low, in the ng/L range, it is necessary to introduce as much sample as possible onto the column without negatively affecting the chromatography or the instrument. Chromatograms from each 0.5, 1.0, and 2.0 µL injection volumes (Figures 4-6) were evaluated to determine if increasing the injection volume increased the abundance of certain compounds while maintaining Gaussian peak shapes and adequate compound separation. It was determined that

increasing the injection volume to 2 µL increased peak abundances while maintaining quality chromatography.

Additionally, since a splitless injection was being performed at a 2 µL injection volume a splitless liner packed with glass wool was used in the instrument's inlet. The glass wool adds more surface area for the injected sample to spread across increasing the volatility. To further increase volatility of the larger injection volume the inlet was set at 250ºC, hot enough to volatilize the entire sample but not so hot as to degrade analytes of interest.

Helium was utilized as the carrier gas for GC/MS operation. Typical flow rates when using helium as the carrier gas range from 1-2 mL/min. Using a lower flow rate allows for analytes to interact with the column's stationary phase longer to increase separation. A higher flow rate will not hurt the instrument but will decrease the sensitivity. A 1 mL/min constant flow rate was used for this method. The purge flow was determined based on what was necessary to clear the inlet of un-volatilized residual sample in order to prevent a secondary sampling. A purge rate of 10 mL/min after one minute of sampling proved to be inadequate (Figure 7) allowing for dual sampling and two peaks resulting for each compound. Increasing the purge flow rate to 30 mL/min after a sampling time of one minute remedied this issue (Figure 8).

Analysis was carried out on a 30-meter 5% phenyl siloxane column with a diameter of 0.25 mm and a film thickness of 0.25 µm. This was chosen as this style of column has been shown to exhibit high quality chromatography in regards to drug analysis and offers a high temperature limit (400ºC). This allowed for an oven temperature program to increase to, and be held at, 300ºC for an extended time with minimal column bleed. The oven program ramp was from 70-300ºC at 15ºC/min to allow for sufficient analyte-stationary phase interaction without having the method be inefficiently long.

The mass spectrometer was capable of operating in both chemical ionization (CI) and electron impact (EI) ionization modes, and EI was selected for this study. The MS quadrupole was set to scan a range that would encompass all drugs of interest. Drugs are small molecules therefore a tight scan range of 35-550 m/z could be set. The instrument is operating in full scan mode with this ion range as this is to be an untargeted method for the detection of all drugs. Increasing the scan range can have a number of effects on the instrument. Scanning too high of a m/z results in the detection of unwanted compounds and scanning too low results in many atmospheric interferences. Also, the larger the scan range the slower the detector can scan this range and can result in decreased sensitivity. The scan rate of the detector is equal to how fast the detector is able to scan the entire selected mass to charge range, or the inverse of a dwell time. The dwell is the amount of scans the detector is able to perform in a single second. Thus, a dwell of 20 means 20 scans per second or equal to a scan rate of 0.05 seconds. Utilizing too low of a scan rate can result in missed ions or analytes. However scanning at too high of a scan rate can result in poor chromatographic peak shape (Figure 9) and loss of sensitivity. It is necessary to optimize the scan rate so that no analytes are missed while maintaining Gaussian peaks (Figure 10). All parameters of the GC/MS need to be optimized per the analysis being performed in order to produce the best chromatography and detection of analytes of interest.

Results and Discussion

Column Capacity

The 25, 50, 75, and 100 mL samples used to evaluate the columns binding capacity all showed an increase in retention proportional to their increased sample size. The resulting column capacity curve (Figure 11) demonstrated that even with the 100 mL sample the column

binding did not reach its maximum capacity. Column capacity curves rise sharply as the amount of analyte increases before plateauing at its maximum binding efficiency. The experimental column capacity curve produced (Figure 11) shows that the binding is still rising with the increased sample volumes. Therefore, it was determined that an even larger sample volume could be used, settling on a sample size of 500 mL. The 500 mL influent sample volume is larger than that of most of the developed targeted methods that utilize sample volumes ranging from 5-250 mL of influent but increase to 500 mL when analyzing effluent samples. Targeted liquid chromatography methods can use decreased sample volumes as they have increased sensitivity when operating in SRM or MRM modes and have the advantage of increased injection volumes.

Solid Phase Extraction Rate

All 1000 ng fortified samples extracted at rates of 1, 5, and 10 mL/min and their resulting recoveries were compared (Figure 12) to determine an optimal extraction flow rate. Calculated nanogram recovery and raw area counts for variable extraction rates were also compared (Table 4; Table 5). Comparing the recovery amounts of the extracted samples to that of the neat standard there was no significant difference between the 1, 5, and 10 mL/min extraction rates. Recovery of methadone in the extracted samples exceeded 100% when compared to the calibration curve but the standard deviation and bias are within 10% of the true value. This amount of fluctuation occurs due to random and systematic errors throughout the extraction and instrumental analysis and therefore it is expected. Based upon this data, future sample extractions could be performed at 10 mL/min increasing the extraction rate tenfold and reducing the overall extraction time without any loss in analyte recovery.

Gas Chromatography-Mass Spectrometry

Extracted samples were run on a Shimadzu single quadrupole gas chromatograph-mass spectrometer (GC/MS) model GCMS-QP2020 (Shimadzu, Koyoto, Japan) equipped with a Shimadzu AOC-20i autosampler utilizing the following optimized method. Onto a wool packed splitless liner (Shimadzu Part#:221-48876-03), 2 µL of sample is injected onto the instrument installed with an Agilent J&W HP-5MS (30 m × 0.25mm, 0.25 µm) column (Agilent Technologies, Santa Clara, California, USA). Utilizing helium as the carrier gas the instrument operated in constant flow mode at a rate of 1.0 mL/min. The GC oven was programmed as follows: held at the initial temperature of 70°C for 2 min then ramped at 15°C/min to 300°C and held for 10 min for a total run time of 27.33 min. The injection port, transfer line, and source were set at 250°C, 280°C, and 200°C respectively. The MS operated in full scan mode, scanning ions from 35-550 *m/z* at an event time of 0.20 seconds (sec) from 3.50-27.33 minutes. The instrument control and data processing were operated by Shimadzu GCMSsolutions laboratory software.

Relative Retention Times

An SPE extraction used in conjunction with a GC/MS full scan method was used in the effort to detect and quantify over fifty pharmaceuticals (Table 6). To establish relative retention times (RRT) for each analyte, neat standards of every compound were run independently. RRTs were calculated in reference to the methadone-d9 internal standard (analyte/IS) run with each analyte. The resulting RRTs for each compound can be seen in Table 7. This is necessary as this is an untargeted method for the analysis of drugs in wastewater and previously developed

targeted methods are focused on a limited number of analytes and can optimize separation of these compounds whereas in an untargeted method analytes may overlap.

Extracted Sample Analysis

Samples carried out through the SPE extraction were run under the developed GC/MS method. Although both basic and acidic fractions were collected, acidic fractions exhibited poor chromatography and the acidic and neutral compounds were lost in the resulting chromatograms. This may be due to the acid/neutral fraction being eluted using a 50:50 (*v/v*) solution of hexanes and ethyl acetate. The SPE column is washed with hexanes prior to this elution step in order to remove any bound proteins and lipids, but any that still remain will be eluted with the 50:50 mixture along with the analytes of interest. These excess lipids cause interference when analyzing the extractions on the GC/MS. The basic fraction produced more reliable chromatography with few overlapping compounds (Figure 12). Not all drugs were present and identifiable in the basic fraction limiting the number of analytes used for the development of calibration curves and quantitation. Therefore, the final number of analytes used for quantitation purposes was 42. However, due to the method being untargeted and utilizing the mass spectrometers full scan setting, all other analytes present in a sample will still be detected but not quantitated. Identification of compounds in the calibrator mix were made based upon RRT and a positive library match within the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) or the National Institute of Standards and Technology (NIST) libraries. For compounds not in the calibration mix, identification was made solely with the use of MS libraries.

Calibration Curves

Calibration curves for the 42 identifiable analytes were constructed with calibrators at concentrations of 0.1, 0.2, 0.4, 1.0, 2.0, 3.0, and 4.0 ng/mL (Figures 4-45). Since the MS was run in scan mode and some analytes in the calibration mix overlapped with one another (Figure 13), extracted ion chromatograms were used (Figure 14) to quantitate these compounds. Additionally, no background correction was performed in order to eliminate the possibility of removing low level analytes buried in the baseline. Calibration curves were constructed based upon a single quantification ion, usually the base peak unless interference was detected. Ions used for quantification are listed in Table 8. No qualifier ions were used for identification as IDs were made as previously described. Calibration curves were plotted with the concentration on the x-axis and an analyte/IS ration on the y-axis. Curves were acceptable if they had a coefficient of determination (R^2) value greater than or equal to 0.9875 and bias in the calibrators less ≤30%. While SWGTOX guidelines allow for 20% bias and some laboratories use 10% bias, ≤30% bias is acceptable for the purpose of monitoring communal drug use since a more stringent acceptable bias would not have a significant impact on the overall results.

Out of 42 drug calibration curves more than 75% of the analytes ran had a passing curve in a single run. Some analytes such as early eluting and late eluting compounds need refining in order to make curves acceptable. Possibly increasing the calibrator concentrations of those analytes with failing curves have low responses even at higher concentrations so that the lower calibrators are better detected. This low abundance in lower calibrators of these compounds results in having high bias (>30%) and reduces the curves R^2 values. For early and late eluting compounds the addition of more deuterated internal standards closer in retention time to these analytes may improve their bias. All compounds in this method reference methadone-d9 as the internal standard which is located in the middle of the chromatogram. Since the single IS is in

the middle of the chromatogram it does not experience the same errors and interferences as those eluting much earlier or later in the chromatogram. Adding two additional internal standards such as methamphetamine-d5 for use with early eluting compounds and buprenorphine-d4 for the late eluting compounds will aid in correcting some of the biases that occur when referencing a distant internal standard.

Current methods for wastewater analysis are developed to analyze an isolated panel of drugs. A majority of these utilize liquid chromatography (Table 2) in either SRM or MRM modes and are incapable of being utilized as a tool for untargeted monitoring of community drug use. Gas chromatography methods have been developed for wastewater analysis (Bisceglia et al., 2010; Logarinho et al., 2016; Racamonde et al., 2012; Shao et al., 2020; Thomas et al., 2004) have similar issues as LC methods. These GC methods use SIM mode to focus on a limited scope of analytes, potentially missing several compounds outside their detection parameters. González-Mariño et al. (2010) developed a GC/MS method for wastewater analysis to operate in scan mode. However, the method does not use the scan mode setting to its fullest potential, scanning only for small ranges of ions across short time windows. Essentially, the method is a modified, compound specific scan method that limits detection to a short list of stimulant and opioid compounds used in the calibration standards. Even with this limited scope of analytes, the González-Mariño method has calibration curves from 5-500 ng/mL, which are far less sensitive than the 0.10-4.0 ng/mL curves developed in the method developed in this study. Additionally, all the GC methods found in the peer-reviewed derivative analysis, increases analytical cost and sample preparation time. Derivatization can increase the sensitivity of many metabolites but does not improve the detection of parent compounds since the derivatizing compounds do not react with the parent analytes. None of the current wastewater methods found

in the literature propose a true untargeted full scan method, thus, are insufficient for comprehensive monitoring of communal drug use.

Conclusion

Wastewater analysis is a proven effective way for monitoring community drug use and has many benefits to the public. Monitoring public use allows for the establishment of drug specific education and treatment programs in the affected areas. Focusing more on prevention and education versus traditional punitive methods of dealing with a drug epidemic. Economical and efficient monitoring of wastewater also keeps law enforcement and medical professionals on top of current drug trends allowing for the proper treatment of overdose victims. With the current state of the drug epidemic the current method of self-reporting surveys, overdose monitoring, and arrest records are longer viable options. Waiting 2-3 years for biased data is too long to wait for outdated unreliable data. With the emerging drug trends of new novel psychoactive substances being produced faster than forensic scientists can keep up, an untargeted GC screen for processing wastewater can help scientists better keep up with those who need help.

This research has demonstrated that it is possible to create a reliable full scan screening method via GC/MS for the detection and quantitation of pharmaceuticals. Additionally, this method uses an increased extraction rate which lends itself to more efficiently extracting large samples. It has greater efficiency then other GC/MS methods that require derivatization and many of those focus solely on a smaller panel of drugs. Gas chromatography and its methods are more cost efficient than their LC counterparts and can be just as reliable for analyzing wastewater.

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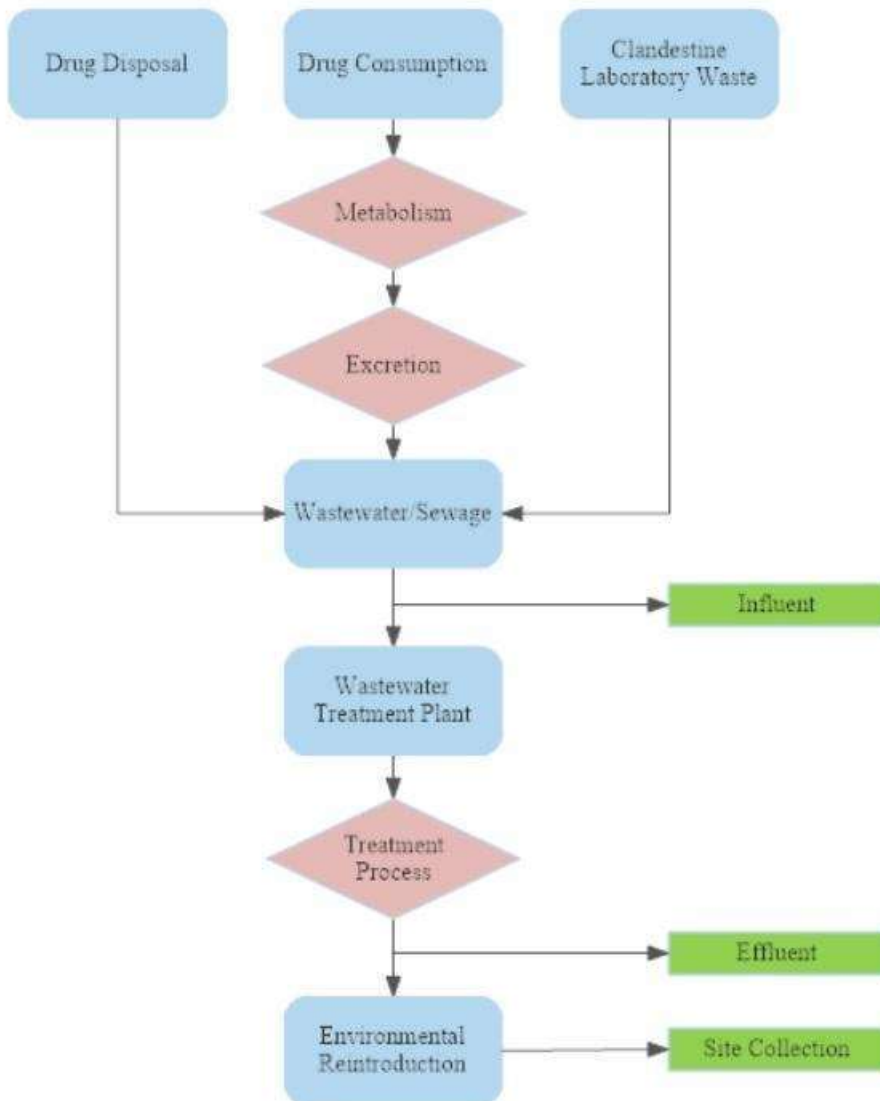
Appendix

Figure 1: Schematic of wastewater deposition and collection.

Table 1: Solid phase extraction schemes for the analysis of influent wastewater. The location under author name is the location the analysis was conducted, which may be different from the sample collection location.

Reference	Analytes	Recovery	Sample Volume	Extraction Scheme
Andrés-Costa et al. (2014) Valencia, Spain	(8): 6-Monoacetylmorphine Amphetamine Benzoylecgonine Cocaine Ketamine Methamphetamine MDMA THC-COOH	500 ng/L 80±17 94±5 92±9 76±20 96±7 92±2 101±6 100±20	250 mL	Phenomenex Strata-X Column (6 mL/500 mg): (Condition: 6 mL MeOH, 6 mL DI Water) (Sample: Filtered/Unbuffered @ 10 mL/min) (Wash: 6 mL DI Water) (Elution: 6 mL MeOH)
Borova et al. (2014) Athens, Greece	(68): 2-Oxo-3-hydroxy-LSD 6-Monoacetylmorphine 7-amine-flunitrazepam 8-OH mirtazapine 9-OH risperidone Alprazolam Amitriptyline Amphetamine Benzoylecgonine Bromazepam Buprenorphine Carbamazepine Chlordiazepoxide Chlorpromazine Citalopram Clobazam Clomipramine Clozapine Cocaine Codeine Diazepam Doxepin Ecgonine methyl ester EDDP Ephedrine Fentanyl Flunitrazepam Fluoxetine Imipramine Ketamine Lamotrigine Levetiracetam Lidocaine Lorazepam LSD MDA MDEA MDMA Methadone Methamphetamine Midazolam Mirtazapine Morphine Nitrazepam	1000 ng/L 105±5 58±18 82±5 88±5 87±9 84±6 90±2 85±3 89±2 76±3 83±19 97±2 88±6 79±8 91±9 96±2 88±2 88±6 88±4 93±10 91±4 94±9 99±11 94±16 88±4 92±2 71±5 91±10 87±7 87±4 91±2 55±7 89±4 45±14 84±7 86±5 89±2 87±3 91±4 85±2 81±14 92±2 119±10 101±20	50 mL	Phenomenex Strata-XC Column (6 mL/250 mg): (Condition: 6 mL MeOH, 6 mL Acidified DI Water pH 2.5) (Sample: Filtered/Buffered pH 2.5 by Gravity) (Wash: 3 mL Acidified DI Water) (Elution: 6 mL 98:2 MeOH:NH3

	Norclozapine	79±11		
	Nordiazepam	87±11		
	Norephedrine	80±11		
	Norfentanyl	90±8		
	Norketamine	86±9		
	Norsertraline	74±19		
	Nortriptyline	94±19		
	Olanzapine	66±6		
	Oxycodone	83±12		
	Oxazepam	49±17		
	Paroxetine	80±4		
	Pentobarbital	87±8		
	Phenobarbital	93±11		
	Phenytoin	86±24		
	Primidone	102±4		
	Risperidone	91±5		
	Sertraline	87±4		
	Temazepam	85±13		
	THC	26±4		
	THC-COOH	22±9		
	Thiopental	89±41		
	Topiramate	99±15		
	Valproic acid	142±30		
	Venlafaxine	92±10		
Burgard et al. (2013) Tacoma, WA, USA	(2): Amphetamine Ritalinic Acid	100 ng/mL 60±20 70±20	100 mL	Waters Oasis MCX Column (3 mL/60 mg): (Condition: 2 mL MeOH, 3 mL Acidified DI Water pH 2) (Sample: Filtered/Buffered pH 2 @ 5 mL/min) (Wash: 2 mL 2% Formic acid, 3 mL MeOH) (Elution: 5 mL 98:2 MeOH:NH$_4$OH)
Carpinteiro et al. (2012) Santiago de Compostela, Spain	(10): 2-benzothiazolamine 2-hydroxybenzothiazole 2-methylbenzothiazole 2-Methylthiobenzothiazole 4-methylbenzotriazole 5-methylbenzotriazole 5,6-dimethyl-1H-benzotriazolebenzothiazole benzothiazole benzotriazole Mercaptobenzothiazole	10 ng/mL 82±11 87±11 87±11 86±12 81±12 81±12 91±11 80±10 83±9 99±11	50-500 mL	Waters Oasis Max Column (150 mg): (Condition: MeOH, DI Water) (Sample: Filtered/Unbuffered @ 5 mL/min) (Elution: 5 mL 70:30 MeOH:Acetone)

Causanilles et al. (2017) Amsterdam, Netherlands	(32): 2,5-dimethoxy-4-bromophenethylamine 4-fluoroampetamine 6-monoacetylmorphine Amphetamine Benzoylecgonine Caffeine Clozapine Cocaine Cotinine Diazepam Ecgonine methyl ester EDDP Fentanyl HMMA Hordenine Ketamine LSD mCPP MDA MDMA Methadone Methylhexanamine Methylphenidate MMA L-759633 Nordiazepam Oxazepam Paraxanthine Ritalinic acid Temazepam THC-COOH Venlafaxine	N/A	50 mL	Waters Oasis HLB Column (6 mL/150 mg): (Condition: 8 mL MeOH, 8 mL DI Water) (Sample: Filtered/Unbuffered) (Wash: 4 mL DI Water) (Elution: 8 mL MeOH)
Celma et al. (2019) Castelló, Spain	(22): 3,4-dimetthoxy-α-PVP 4-C-α-PPP 4-FMC 4-MEC 4-MePPP α-PVP Amphetamine Benzoylecgonine Butylone Cocaine Dimethylone Dipentylone Ketamine MDMA MDPV Mephedrone Methamphetamine Methedrone Methoxetamine Methylone N-ethcathinone PMMA	1000 ng/L 99±7 84±8 92±9 86±5 85±5 95±11 117±6 90±3 95±3 114±2 97±5 107±6 92±3 100±2 96±7 112±3 119±4 94±5 105±5 103±5 88±7 99±5	5 mL	Waters Oasis HLB Column (3 mL/60 mg): (Condition: 6 mL MeOH, 6 mL DI Water) (Sample: Unfiltered/Unbuffered) (Wash: 50 mL DI Water) (Elution: 1 mL MeOH)

Reference / Location	Analytes	Recovery (%)	Sample Volume	SPE Method
Damien et al. (2014) Schoelcher Cedex, France	(17): 6-monoacetylmorhine Amphetamine Benzoylecgonine Buprenorphine Cocaethylene Cocaine Ecgonine methyl ester EDDP Heroin MDA MDEA MDMA Methadone Methamphetamine Morphine Norcocaine THC-COOH	N/A	200 mL	Waters Oasis HLB Column (6 mL/500 mg): (Condition: 10 mL MeOH, 10 mL DI Water) (Sample: Filtered/Unbuffered @ 2 mL/min) (Wash: 10 mL DI Water) (Elution: 10 mL MeOH)
González-Mariño et al. (2012) Santiago de Compostela, Spain	(24): 2-Oxo-3-hydroxy-LSD 6-monoacetylmorphine Amphetamine Benzoylecgonine Benzylpiperazine Cocaethylene Cocaine Codeine EDDP Fentanyl Heroin Ketamine LSD mCPP MDA MDEA MDMA Methadone Methamphetamine Morphine PCP Scopolamine THC THC-COOH	500 ng/L 91.6 ± 7.4 94.9 ± 11.7 111.7 ± 7.0 121.8 ± 12.7 100.9 ± 8.6 119.3 ± 3.3 94.3 ± 4.2 94.3 ± 22.1 62.9 ± 7.7 109.9 ± 3.5 99.0 ± 29.7 115.8 ± 4.7 103.4 ± 3.9 80.4 ± 15.5 114.2 ± 12.2 115.4 ± 7.3 111.4 ± 9.8 108.4 ± 4.3 91.5 ± 15.3 130.8 ± 22.8 111.6 ± 8.1 100.0 ± 6.1 105.4 ± 20.5 107.4 ± 10.8	200 mL Influent 500 mL Effluent	Waters Oasis MCX Column (6 mL/500 mg): (Condition: 2 mL 95:5 MeOH:NH4OH, 2 mL Acidified DI Water pH 4.5) (Sample: Filtered/Buffered pH 4.5 @ 10 mL/min) (Wash: 10 mL DI Water) (Elution: (Acidic) 2 mL MeOH, (Basic) 4 mL 95:5 MeOH:NH4OH)
Gul et al. (2016)a Gul et al. (2016)b Oxford, MS, USA	a(7): Amphetamine Benzoylecgonine Cocaine MDA MDEA MDMA Methamphetamine b(8): 6-Monoacetylmorphine Codeine, Hydrocodone Hydromorphone Morphine Norhydrocodone Oxycodone Oxymorphone	86.1-112.6 $\pm$ 0.23-0.43 N/A	15 mL	Phenomenex Strata Screen-C (3 mL/150 mg): (Condition: 2 mL MeOH, 2 mL DI Water) (Sample: Filtered/Unbuffered) (Wash: 9 mL 0.1N HCl, 6 mL MeOH) (Elution: 2 mL 99:1 MeOH:NH$_3$)

Reference	Analytes	Recovery	Volume	SPE Method
Hass et al. (2011) Berlin, Germany	(7): Diazepam Meprobamate Oxazepam Phenobarbital Phenylethylmalonamide Primidone Pyrithyldione	1000 ng/L 84±11 94±4 91±6 59±16 52±21 90±11 84±5	100 mL Influent 250 mL Effluent	Mallinckrodt Baker RP-C18 (6 mL/500 mg): (Condition: 4 mL 80:20 MeOH:Acetone, 4 mL MeOH, 4 mL DI Water) (Sample: Filtered/Unbuffered @ 15 mL/min) (Wash: 4 mL DI Water) (Elution: 4 mL 80:20 MeOH:Acetone)
Kinyua et al. (2014) Antwerp, Belgium	(7): Butylone Ehylone Methiopropamine Methoxetamine Methylone PMA PMMA	100 ng/L 96±2 95±10 96±3 99±3 105±1 94±7 97±3	50 mL	Waters Oasis MCX Column (3 mL/60 mg): (Condition: 6 mL MeOH, 4 mL DI Water, 4 mL Acidified DI Water pH 2) (Sample: Filtered/Buffered pH 2 @ 2.5 mL/min) (Wash: 3 mL DI Water) (Elution: 2 mL MeOH, 2 mL 95:5 MeOH:NH4OH)
Li et al. (2014) Beijing, China	(2): Amphetamine Methamphetamine	85-105	50 mL	Waters Oasis MCX Column (3 mL/60 mg): (Condition: MeOH, DI Water, Acidified DI Water pH 2)(Sample: Filtered/Unbuffered) (Wash: 4 mL DI Water) (Elution: 4 mL 95:5 MeOH:NH3)
Ling-Hui et al. (2014) Beijing, China	(30): Amitriptyline Bupropion Chlorpromazine Citalopram Clomipramine Clozapine Demethylclozapine Desmethylfluvoxamine Desmethylmirtazapine Desmethylvenlafaxine Doxepin Duloxetine Fluoxetine Fluphenazine Fluvoxamine Imipramine Mianserin Milnacipran Mirtazapine Moclobemide Norclomipramine Nordoxepin Norfluoxetine Nortriptyline Olanzapine Paroxetine Quetiapine Sertraline Trazadone Venlafaxine	1000 ng/L 81.2-118	300 mL	3M Empore 47mm Cation Disk: (Condition: 8 mL MeOH, 8 mL DI Water, 8 mL acidified DI Water pH 3) (Sample: Filtered/Buffered pH 3 by Gravity) (Wash: 10 mL DI Water, 10 mL 90:10 DI Water:MeOH) (Elution: 12 mL 92:8 MeOH:NH$_3$)

Logarinho et al. (2016) Covilhã, Portugal	(6): Chlorpromazine Clozapine Cyamemazine Haloperidol Levomepromazine Quetiapine	1000 ng/L 45.3±6.7 46.2±1.1 67.5±3.8 75.8±7.5 34.1±1.4 72.6±6.6	50 mL	Phenomenex Strata-X Column (250 mg): (Condition: 2 mL MeOH, 2 mL Acidified DI Water pH 5) (Sample: Filtered/Unbuffered) (Wash: 2 mL 0.1N HCl, 2 mL 0.1N HCl in MeOH) (Elution: 6 mL MeOH) (Elution: 2 mL 95:5 MeOH:NH$_3$)
Mosekiemang et al. (2019) Matieland, South Africa	(10): 8,14-dihydroxy-efavirenz 12-hydroxy-nevirapine Desthiazolylmethyloxycarbonyl Rironavir Efavirenz Emtricitabine Lamivudine Nevirapine Rironavir Zidovudine Zidovudine glucuronide	N/A	50 mL	Phenomenex Strata SDB-L Column (6 mL/200 mg): (Condition: 4 mL CAN, 4 mL MeOH, 4 mL DI Water pH 7) (Sample: Filtered/Buffered pH 7 @ 1 mL/min) (Elution: 4 mL 49:49:2 MeOH:DCM:Formic acid)
Nefau et al. (2013) Paris, France	(16): 6-Monoacetylmorphine Amphetamine Benzoylecgonine Buprenorphine Cocaethylene Cocaine Ecgonine methyl ester EDDP Heroin MDA MDEA MDMA Methadone Morphine Norcocaine THC-COOH	400 ng/L 124±37 104±37 119±54 109±93 109±41 107±36 110±24 103±66 121±82 103±36 110±23 113±23 92±37 79±64 105±34 61±57	250 mL Influent 500 mL Effluent	Waters Oasis HLB Column (6 mL/500 mg): (Condition: 10 mL MeOH, 10 mL DI Water) (Sample: Filtered/Unbuffered @ 2 mL/min) (Wash: 10 mL DI Water) (Elution: 10 mL MeOH)

Salgueiro-González et al. (2019) Milan, Italy	(40) 1-Cyclohexyl-4-(1,2-diphenylethyl)piperazine 23-I-NBOMe 2C-B-NBOMe 2C-C-NBOMe 2-Chloro-4,5-methylendioxy-methylamphetamine 2C-iP-NBOMe 2-Phenethylamine 3,4-Dimethoxy-α-pyrrolidinovalerophenone 3,4-dimethylmethcathinone 3-methoxymethcathinone 3-methylmethcathinone 4,4-Dimethylaminorex 4′-Chloro-α-Pyrrolidinopropiophenone 4-fluoromethcathinone 4-Methylaminorex 4-methylethcathinone 5-(2-Aminopropyl)indole 5-Fluoropentyl-3-pyridinoylindole 5-Methoxy-N,N-diallyltryptamine 5-Methoxy-N-isopropyl-N-methyltryptamine 6-(2-Aminopropyl)-benzofuran Buphedrone Butylone Dipentylone Ethcathinone MDMB-CHMICA Mephedrone Methcathinone Methedrone Methoxetamine MDPV Methylone N-ethyl-1,2-diphenylethylamine PMA PMMA Pentedrone Pentylone α-Methyltryptamine α-pyrrolidinovalerophenone α-pyrrolindinopentiothiophenone	N/A	50 mL	Waters Oasis HLB Column (3 mL/60 mg): (Condition: 6 mL MeOH, 3 mL DI Water) (Sample: Filtered/Buffered pH 7 @ 5 mL/min) (Elution: 4 mL MeOH) Waters Oasis MCX (6 mL/150 mg) (Condition: 10 mL MeOH, 5 mL DI Water, 5 mL Acidified DI Water pH 2) (Sample: Filtered/Buffered pH 2 @ 5 mL/min) (Elution: 2 mL 98:2 MeOH:NH$_3$)
Shao et al. (2020) Dalian, China	(2): Amphetamine Methamphetamine	1000 ng/L 76±32 109±23	60 mL	Waters Oasis MCX Column (3 mL/60 mg): (Condition: 6 mL MeOH, 4 mL DI Water, 4 mL Acidified DI Water pH 2) (Sample: Unfiltered/Buffered pH 2) (Elution: 4 mL 96:4 MeOH:NH$_3$)

Stamper et al. (2016) Stamper et al. (2017) Oxford, MS, USA	2016 (8): EDDP Fentanyl Meperidine Methadone Norfentanyl Normeperidine PCP Tramadol	89-110 ± 1.8-9.7	30 mL	Phenomenex Strata Screen-C (3 mL/150 mg): (Condition: 2 mL MeOH, 2 mL DI Water) (Sample: Filtered/Unbuffered) (Wash: 9 mL 0.1N HCl, 6 mL MeOH) (Elution: 2 mL 99:1 MeOH:NH3)
	2017 (10): 2-Hydroxyethyl-flurazepam 7-NH$_2$-flunitrazepam Alprazolam Chlordiazepoxide Flurazepam Nordiazepam Oxazepam Temazepam α-OH-Alprazolam α-OH-Triazolam	N/A		
Vuori et al. (2014) Helsinki, Finland	(11): 6-monoacetylmorphine Amphetamine Benzoylecgonine Codeine EDDP MDMA MDPV Methamphetamine Methadone Morphine THC-COOH	 99-113 82-119 91-144 91-101 86-143 91-119 97-117 100-118 99-128 95-105 93-103	100 mL	Waters Oasis HLB Column (6 mL/200 mg): (Condition: 4 mL MeOH, 4 mL DI Water) (Sample: Unfiltered/Unbuffered @ 10 mL/min) (Wash: 20 mL DI Water) (Elution: 4 mL MeOH)
Yargeau et al. (2014) Montreal, QC, Canada	(18): 6-monoacetylmorphine Acetylcodeine Amphetamine Benzoylecgonine Cocaine Codeine Dihydrocodeine EDDP Ephedrine Heroin Ketamine MDA MDMA Methadone Methamphetamine Morphine Oxycodone Tramadol	83-101	100 mL Influent 200 mL Effluent	Waters Oasis MCX Column: (Condition: 6 mL Acetone, 6 mL MeOH, 6 mL Acidified DI Water pH 2.5 @ 1 ml/min) (Sample: Filtered/Buffered pH 2.5) (Elution: 9 mL 95:5 MeOH:NH$_4$OH)

N/A= Not available or not given, MeOH= Methanol, DCM= Dichloromethane

2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine(EDDP); Lysergic acid diethylamide (LSD); 3,4-Methylenedioxyamphetamine(MDA); 3,4-Methylenedioxy-N-ethylamphetamine(MDEA); 3,4-Methylenedioxymethamphetamine(MDMA); Methylenedioxypyrovalerone (MDPV); Phencyclidine (PCP); para-Methoxyamphetamine (PMA); para-Methoxymethamphetamine (PMMA)

Table 2: Liquid Chromatography-Mass Spectrometry methods for the analysis of pharmaceuticals in wastewater. The location under author name is the location the analysis was conducted, which may be different from the sample collection location.

Liquid Chromatography					Mass Spectrometer		
Reference	Instrument	Column Specific	Mobile Phase	Method	Ionization	MS Type	Mode
Andrés-Costa et al. (2014) Valencia, Spain	Agilent 1260 UHPLC Agilent 1260 Infinity	Phenomenex Kinetex C18 (50 mm×2.10 mm, 1.7 μm)	Mobile A: 0.1% Formic Acid in Water Mobile B: 0.1% Formic Acid in Methanol	Equilibration: 15 min Gradient: 12 min 10%(5)-95%(13) @ 30°C Flow: 0.20 mL/min	ESI (+)	Agilent 6410 QqQ-MS/MS AB Sciex Triple TOF 6400	SRM
Borova et al. (2014) Athens, Greece	Thermo Accela UHPLC	Phenomenex Kinetex PFP (50 mm×2.10 mm, 1.7 μm)	Mobile A: 0.05% Formic Acid in Water Mobile B: 0.05% Formic Acid in Methanol	Positive Mode Equilibration: 16 min Gradient: 20 min 2%(3)-100%(20) @ 25°C Purge: 26 min Flow: 0.10 mL/min Negative Mode Equilibration: 5 min Gradient: 20 min 2%(3)-100%(20) @ 25°C Purge: 7 min Flow: 0.10 mL/min	ESI (+/-)	Thermo TSQ QqQ-MS/MS	SRM
Burgard et al. (2013) Tacoma, WA, USA	Agilent 1290 UHPLC	Phenomenex Kinetex C18 (100 mm×2.10 mm, 2.6 μm)	Mobile A: 0.5% Acetic Acid in Water Mobile B: Acetonitrile	Equilibration: 9 min Gradient: 5 min 0%(5)-40% @ 30°C 1 min 40%-100% @ 30°C Purge: 1 min Flow: 0.35 mL/min	ESI (+)	Agilent 6460 QqQ-MS/MS	SRM
Carpinteiro et al. (2012) Santiago de Compostela, Spain	Varian ProStar 210 HPLC	Zorbax SB-phenyl (100 mm×2.1 mm, 3.5 μm)	Mobile A: 1 mM ammonium acetate in Water Mobile B: 1 mM ammonium acetate in Methanol	Equilibration: 7 min Gradient: 6 min 15%(2)-40% @ 35°C 6 min 40%-70% @ 35°C 3 min 70%-100% @ 35°C Purge: 3 min Flow: 0.15 mL/min	ESI (+)	Varian MS 1200L QqQ-MS/MS	MRM

Causanilles et al. (2017) Amsterdam, Netherlands	Agilent HPLC Thermo HPLC	Phenomenex Biphenyl (100 mm×2.1 mm, 2.6 μm)	Mobile A: 0.04% Formic Acid in Water Mobile B: 0.04% Formic Acid in 80:20 ACN:Water	Equilibration: 3.5 min Gradient: 18 min 2%(2)-40% @ ?°C 7 min 40%-90%(4) @ ?°C Purge: 0.5 min Flow: 0.40 mL/min	ESI (+)	Agilent 6530 QTOF-MS Thermo LTQ-FT Orbitrap	Scan
Celma et al. (2019) Castelló, Spain	Waters Acquity H-Class UPLC	Waters Acquity UPLC BEH C18 (50 mm×2.10 mm, 1.7 μm)	Mobile A: 0.1% Formic Acid in Water Mobile B: 0.1% Formic Acid in Methanol	Equilibration: 5.5 min Gradient: 2 min 10%-60% @ 40°C 0.5 min 60%-90%(1) @ 40°C Purge: 3.6 min Flow: 0.30 mL/min	ESI (+)	Waters Xevo TSQ QqQ-MS/MS	SRM
Damien et al. (2014) Schoelcher Cedex, France	Thermo Accela UHPLC	Waters Xbridge Phenyl (150 mm×3 mm, 3.5 μm)	Mobile A: ACN Mobile B: 5 mM Ammonium Formate in Water pH 4	Equilibration: 5.5 min Gradient: 19 min 98(3)%-10%(2) @ ?°C Purge: 0.5 min Flow: 0.40 mL/min	ESI (+/-)	Thermo Quantum Access Max QqQ-MS	SRM
González-Mariño et al. (2012) Santiago de Compostela, Spain	Agilent 1200 HPLC	Nucleosil 100-3 C18 HD (125 mm×2 mm, 3 μm)	Mobile A: 5 mM ammonium acetate in Water pH 8.5 Mobile B: 5 mM ammonium acetate in Methanol pH 4.5	Equilibration: 10 min Gradient: 24.8 min 2%(0.2)-50% @ 40°C 4 min 50%-100% @ 40°C Purge: 1 min Flow: 0.20 mL/min	ESI (+)	Agilent 6520 QTOF-MS	SRM
Gul et al. (2016)a Gul et al. (2016)b Stamper et al. (2016) Stamper et al. (2017) Oxford, MS, USA	Shimadzu Prominence HPLC	Phenomenex Synergi Hydro-RP column (150mm×3.0 mm; 4 μm)	Mobile A: 0.1% Formic Acid in Water Mobile B: 0.1% Formic Acid in ACN	Equilibration: 4.5 min Gradient: 1 min 2%(2)-20% @ 30°C 5 min 20%-60% @ 30°C 2 min 60%-80% @ 30°C Purge: 0.5 min Flow: 0.65 mL/min	ESI (+)	Applied Biosystems/MSD Sciex Qtrap3200	MRM
Hass et al. (2011) Berlin, Germany	Waters Acquity UPLC	Waters Acquity UPLC BEH C18 (50 mm×2.10 mm, 1.7 μm)	Mobile A: 0.2% Acetic Acid in Water Mobile B: 0.2% Acetic Acid in Methanol	Equilibration: 3 min Gradient: 5.4 min 30%(0.6)-90%(2) @ 40°C Flow: 0.20 mL/min	ESI (+)	Waters Quattro Tandem MS	SRM MRM

Kinyua et al. (2014) Antwerp, Belgium	Agilent 1260 Infinity HPLC	Phenomenex Luna HILIC (150 mm×3 mm, 5 μm)	Mobile A: 5 mM Ammonium Acetate in Water Mobile B: ACN	Equilibration: 4.3 min Gradient: 4.5 min 95%(0.5)-90% @ ?°C 7.5 min 90%-70%(0.1) @ ?°C 2 min 70%-30% @ ?°C Purge: 0.1 min Flow: 0.40 mL/min	ESI (+)	Agilent 6410 QqQ-MS/MS	MRM
Li et al. (2014) Beijing, China	Shimadzu UFLCXR-LC	Phenomenex Luna HILIC (150 mm×3 mm, 5 μm)	Mobile A: 5 mM Ammonium Acetate in Water Mobile B: ACN	? Flow: 0.60 mL/min	ESI (+)	AB Sciex ABI 4000 QqQ-MS/MS	MRM
Ling-Hui et al. (2014) Beijing, China	Agilent 1290 UHPLC	Waters Acquity BEH C18 (150 mm×2.10 mm, 1.7 μm)	Mobile A: 0.1% Formic Acid in 10 mM Ammonium Acetate Mobile B: ACN	Gradient: 10 min 20%(5)-60% @ ?°C Purge: 5 min Flow: 0.30 mL/min	ESI (+)	AB Sciex 5500 QTrap Tandem MS	MRM Scan
Mosekiemang et al. (2019) Matieland, South Africa	Waters Acquity UPLC	Waters Acquity HSS T3 (150 mm×2.10 mm, 1.8 μm)	Mobile A: 0.1% Formic Acid in Water Mobile B: ACN	Equilibration: 3 min Gradient: 8 min 2%(0.2)-98%(0.5) @ 35°C Purge: 0.5 min Flow: 0.30 mL/min	ESI (+)	Waters Xevo TW-S QqQ-MS	MRM
Nefau et al. (2013) Paris, France	Thermo Accela UHPLC	Waters Xbridge Phenyl (150 mm×3 mm, 3.5 μm)	Mobile A: ACN Mobile B: 5 mM Ammonium Formate Buffer pH 4	Equilibration: 5.5 min Gradient: 19 min 98%(3)-10% @ ?°C Purge: 0.5 min Flow: 0.40 mL/min	ESI (+/-)	Thermo Quantum Access Max QqQ-MS	SRM
Salgueiro-González et al. (2019) Milan, Italy	Agilent 1200 HPLC	Waters Xbridge C18 (100 mm×2.1 mm, 3.5 μm)	Mobile A: 0.1% Formic Acid in Water Mobile B: ACN	Equilibration: 6 min Gradient: 20 min 10%-60% @ 30°C 5 min 60%-99%(5) @ 30°C Purge: 1 min Flow: 0.20 mL/min	ESI (+)	Thermo Q-Exactive Hybrid Q-Orbitrap	Scan
Vuori et al. (2014) Helsinki, Finland	Agilent 1200 HPLC	Phenomenex Gemini-NX C18 (100 mm×2 mm, 3 μm)	Mobile A: 10 mM Ammonium Acetate Buffer pH 3.2 Mobile B: 0.1% Formic Acid in Methanol	Equilibration: 10 min Gradient: 0 min 15%-95%(8) @ 40°C Purge: 2 min Flow: 0.30 mL/min	ESI (+)	AB Sciex 4000 QTrap	SRM

Table 3: Gas Chromatography Mass Spectrometry methods for the analysis of pharmaceuticals in wastewater. The location under author name is the location the analysis was conducted, which may be different from the sample collection location.

Gas Chromatograph						Mass Spectrometer		
Reference	Instrument	Injector	Carrier	Column	Oven	Detector	Source Temp	Mode
Bisceglia et al. (2010) Baltimore, MD, USA	Thermo Fisons 8000Top GC	2 µL Splitless @ 240°C Derivatized: BSTFA	?	J&W Scientific DB-5MS (30 m × 0.25mm, 0.25 µm)	105°C(1)-285°C(10) @ 8°C/min	Thermo Fisions MD800 MS	?	SIM
González-Mariño et al. (2010) Santiago de Compostel, Spain	Varian CP3900 GC	2 µL Splitless @ 280°C Derivatized: MSTFA	Helium @ 1.3 mL/min	J&W Scientific HP-5MS (30 m × 0.25mm, 0.25 µm)	90°C(1)-130°C @ 25°C/min - 280°C(5) @ 4°C/min	Varian Saturn 2100 Ion Trap MS	220°C	Scan
Logarinho et al. (2016) Covilhã, Portugal	HP 7890A GC	2 µL Splitless @ 250°C Derivatized: MSTFA/TCMS	Helium @ 0.80 mL/min	J&W Scientific HP-5MS (30 m × 0.25mm, 0.25 µm)	120°C-300°C(14) @ 20°C/min	Agilent 7000B QqQ-MS	280°C	MRM
Racamonde et al. (2012) Santiago de Compostel, Spain	Agilent 7890A GC	3 min Splitless @ 250°C SPME-Derivatized: MSTFA	Helium @ 1.0 mL/min	J&W Scientific HP-5MS (30 m × 0.25mm, 0.25 µm)	90°C(3)-170°C @ 25°C/min - 280°C(5) @ 2°C/min	Agilent 5975C MSD	230°C	SIM
Shao et al. (2020) Dalian, China	Agilent 7890B GC	1 µL Splitless @ 230°C Derivatized: TFA	?	J&W Scientific HP-1 (30 m × 0.25mm, 0.25 µm)	90°C-180°C @ 10°C/min	Agilent 5977A MSD	230°C	SIM
Thomas et al. (2004) Fairfax, VA, USA	HP 5890 GC	1 µL Splitless @ 250°C Derivatized: BSTFA	?	Varian Factor Four-5MS (30 m × 0.25mm, 0.25 µm)	60°C(2)-290(6)°C @ 5°C/min	HP 5971 MSD	290°C	SIM
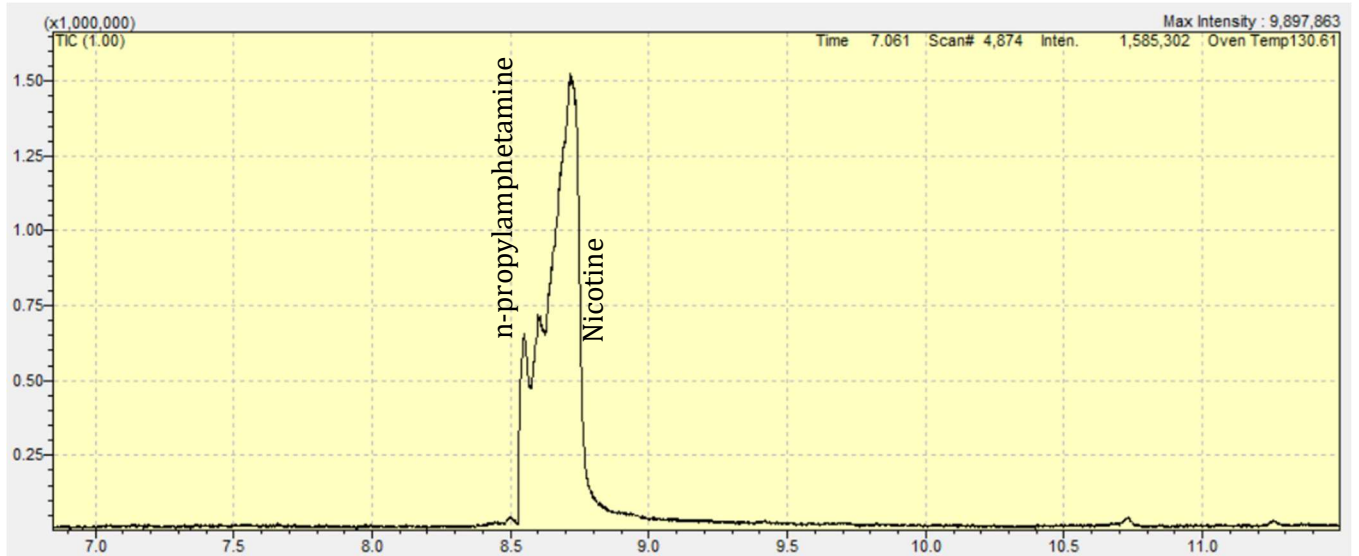
Figure 2: Injection using rubber lined autosampler vial cap.
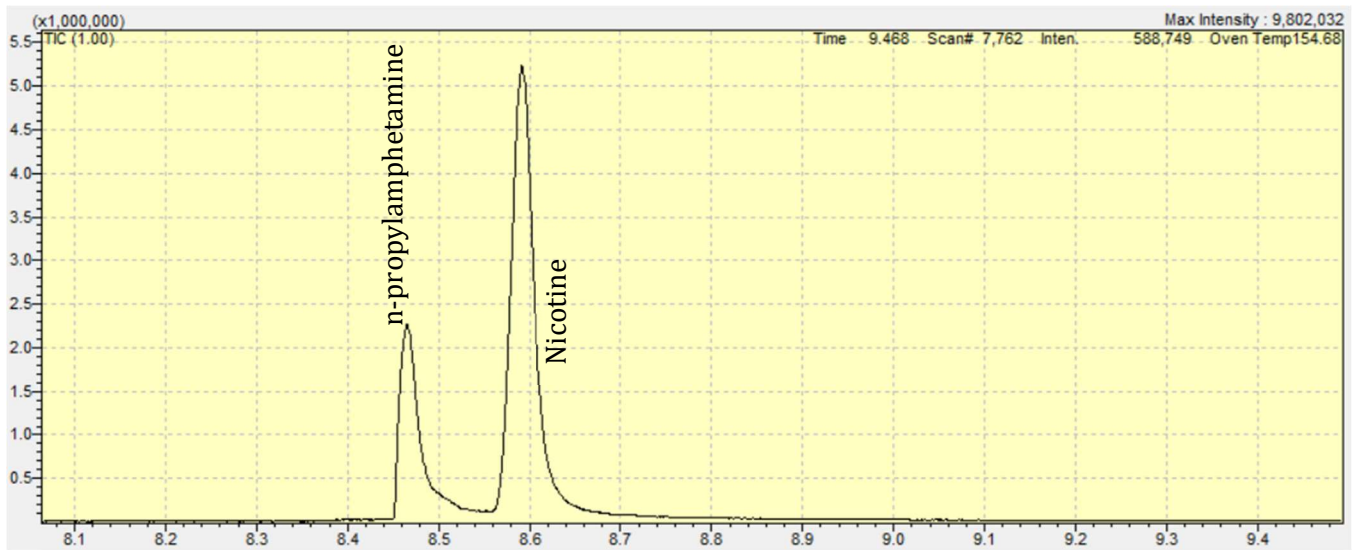
Figure 3: Injection using teflon lined autosampler vial cap.
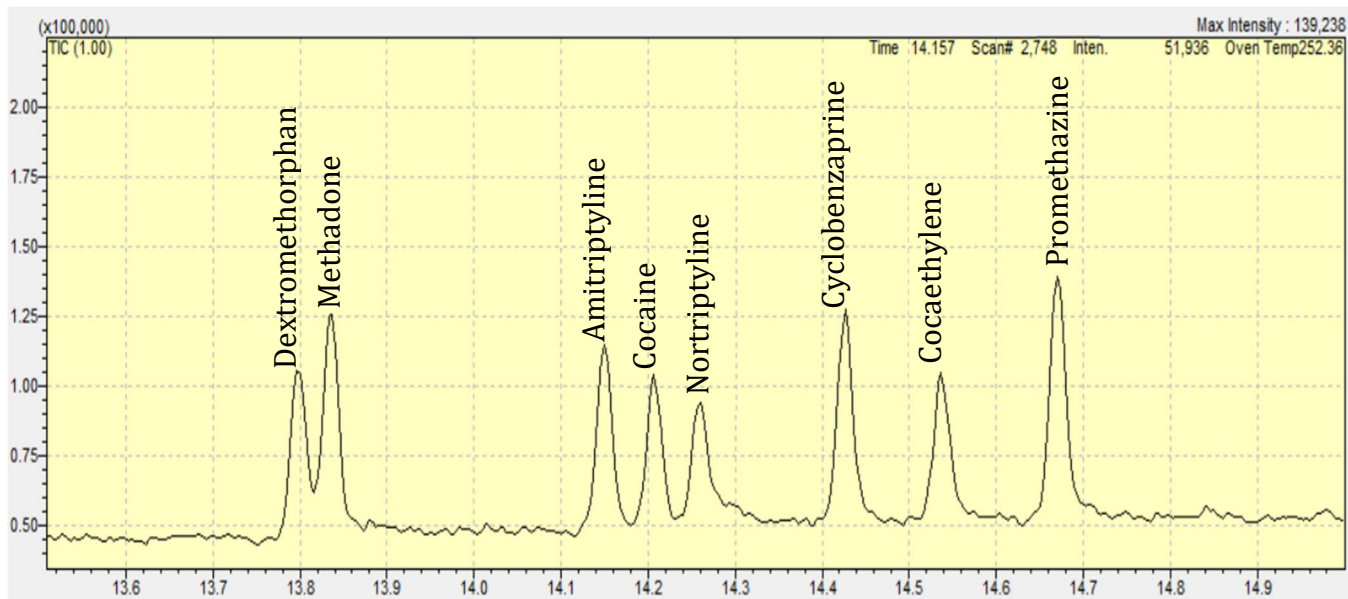
Figure 4: Sample chromatograph using a 0.5 µL injection volume.
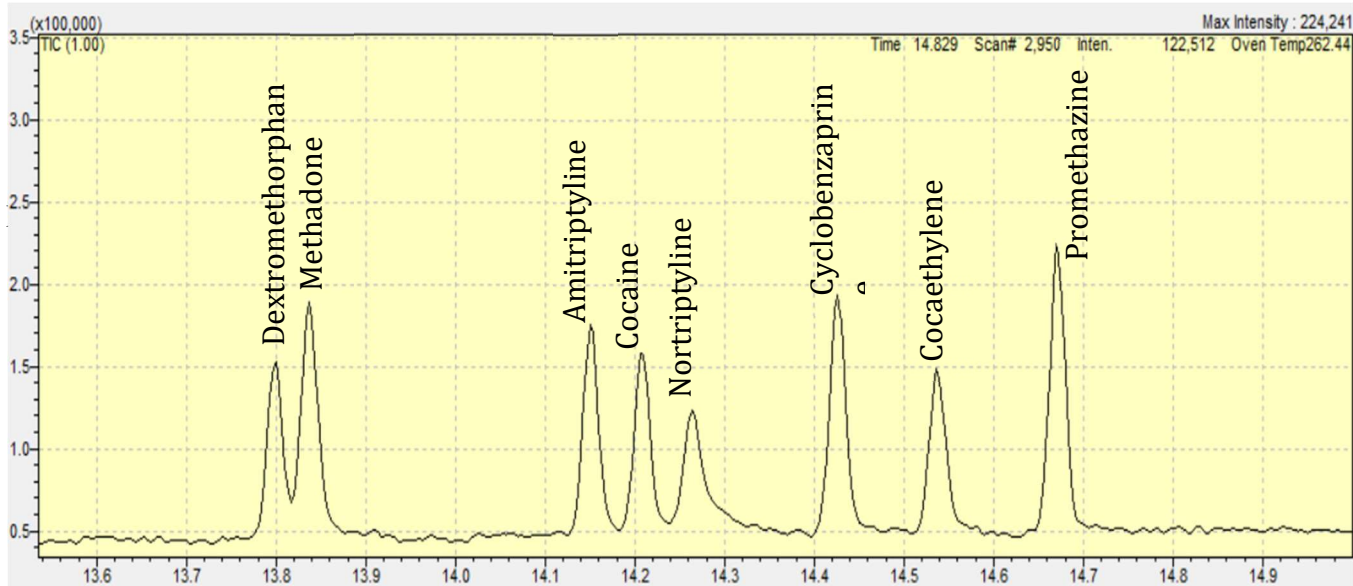
Figure 5: Sample chromatograph using a 1.0 µL injection volume.
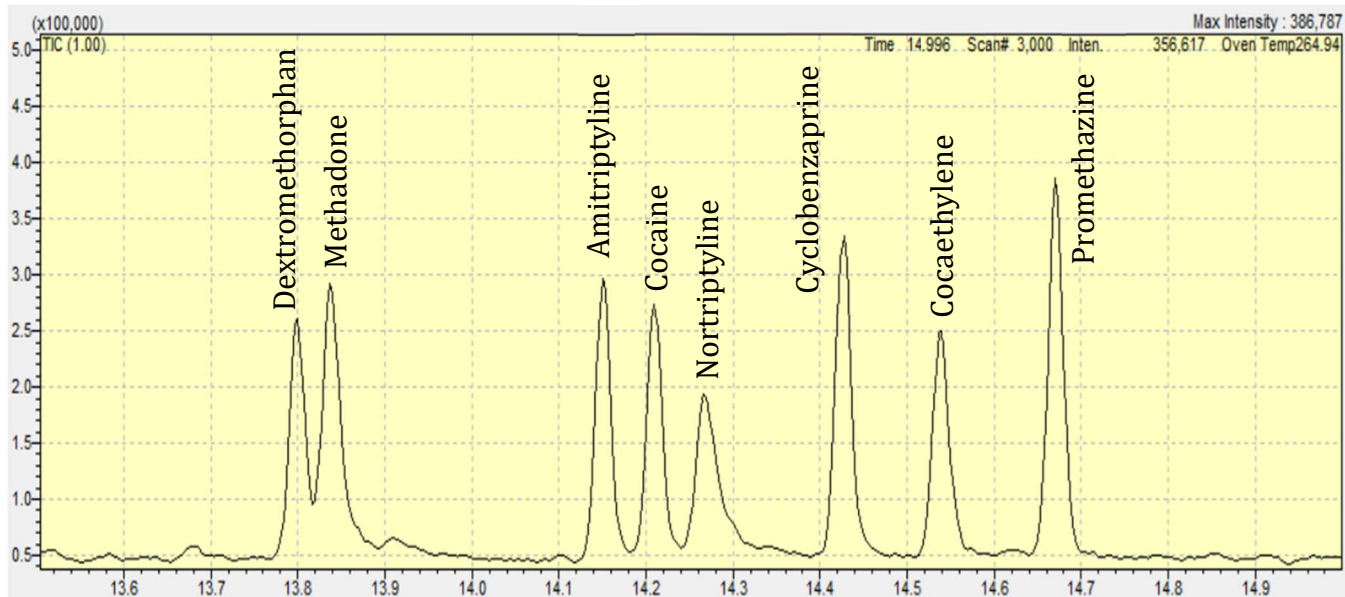
Figure 6: Sample chromatograph using a 2.0 µL injection volume.
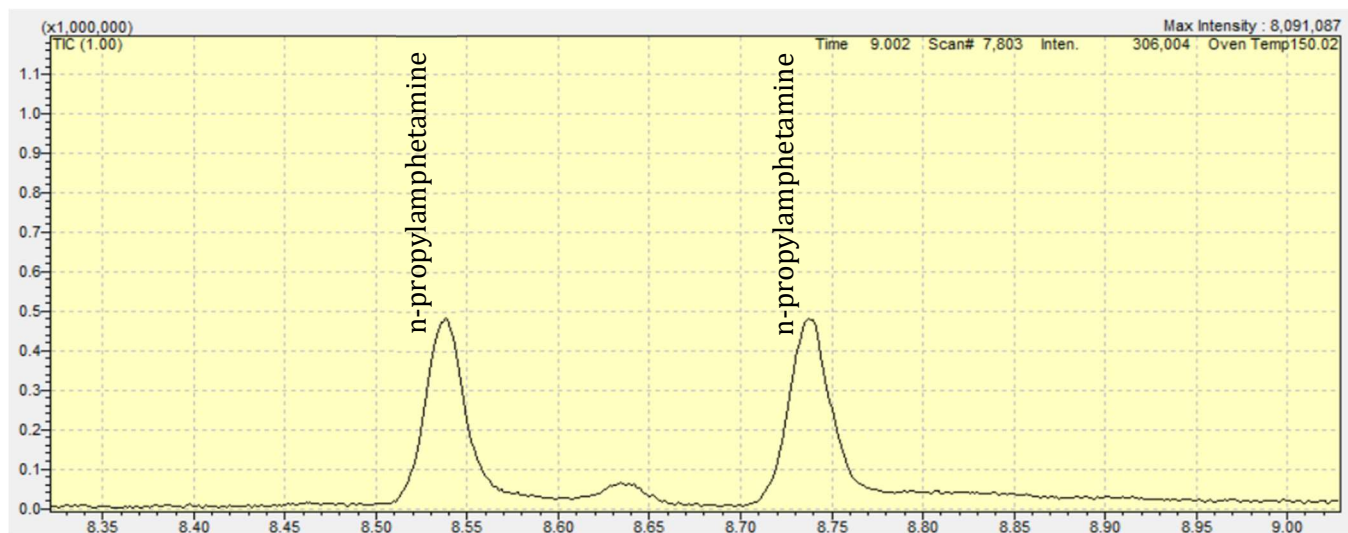
Figure 7: Sample chromatograph with purge flow rate of 10 mL/min after 1 min sampling time.
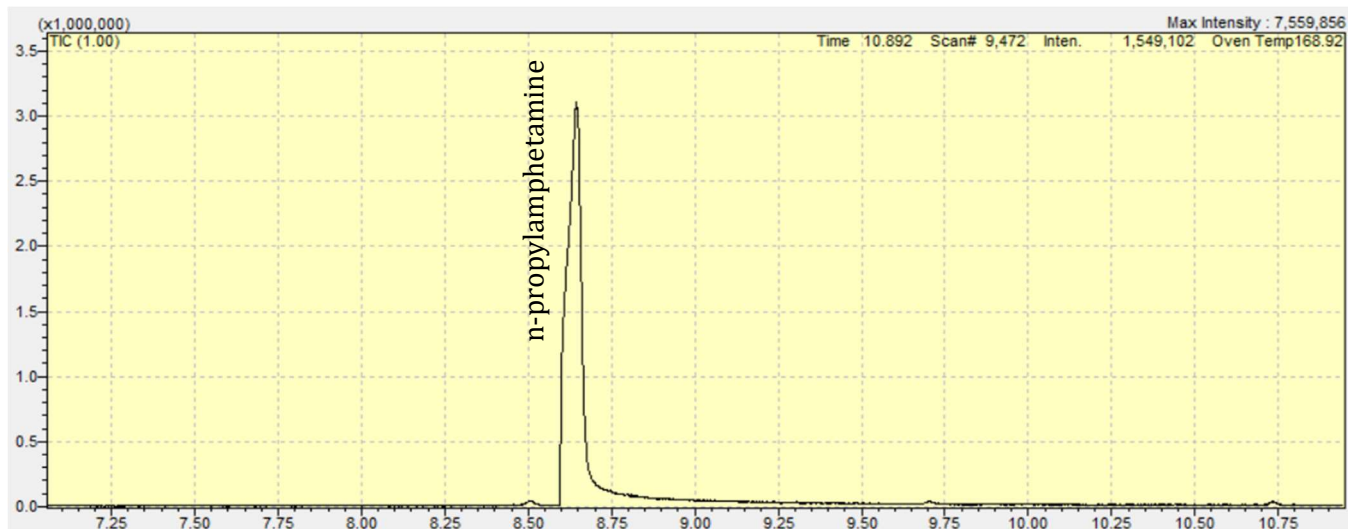
Figure 8: Sample chromatograph with purge flow rate of 30 mL/min after 1 min sampling time.
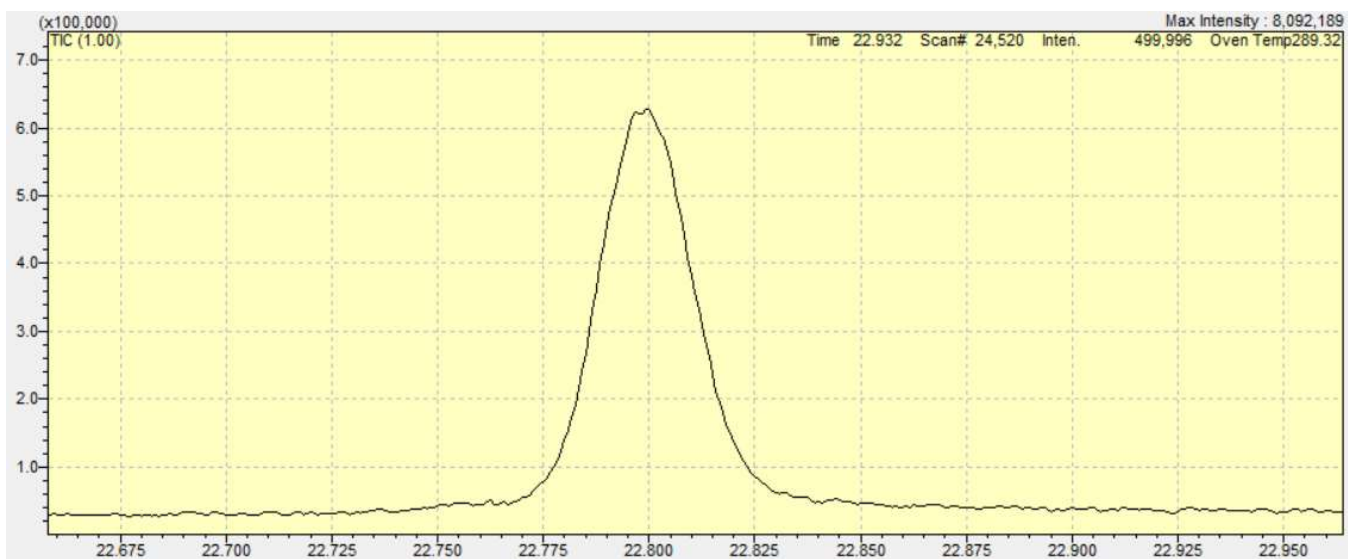
Figure 9: Sample chromatograph utilizing a scan rate of 0.05 seconds

Figure 10: Sample chromatograph utilizing a scan rate of 0.20 seconds
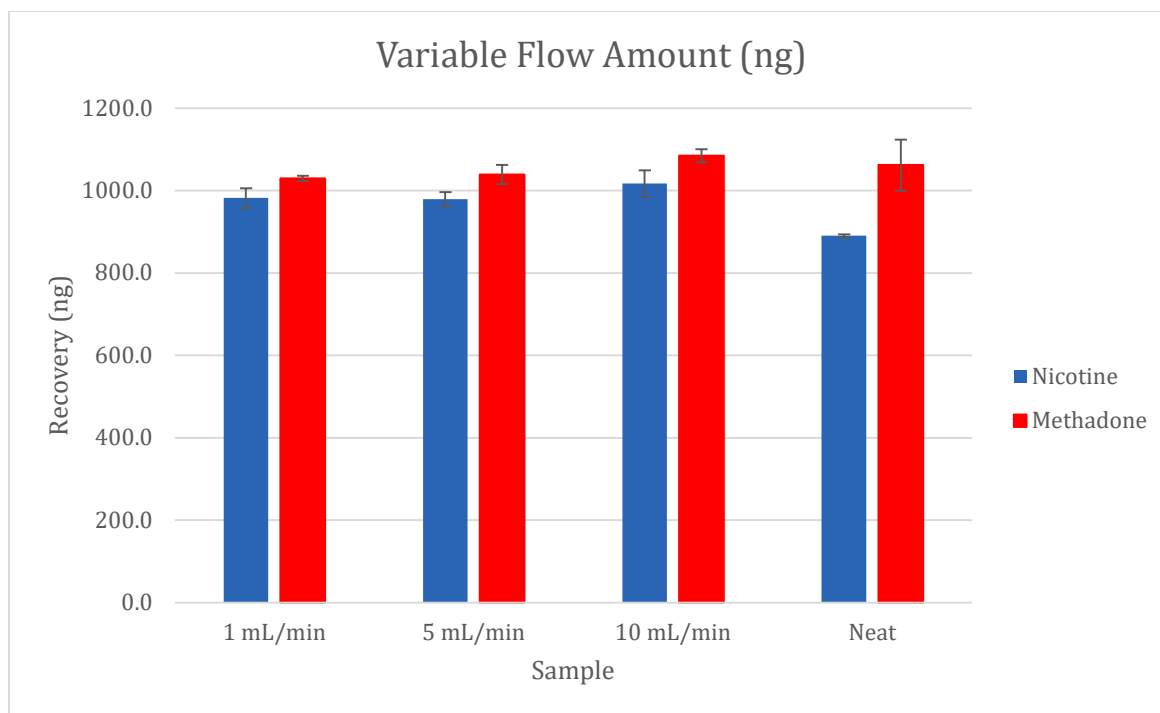
Figure 11: Percent recovery comparison of variable extraction rates using methadone and nicotine fortified at 1000 ng total per sample. (n=3)

Table 4: Calculated value of 1000 ng total fortified samples from variable extraction rates (n=3).

Sample	Nicotine (ng)	Nicotine (STD)	Methadone (ng)	Methadone (STD)
1 mL/min	981.8	24.4	1029.8	6.5
5 mL/min	979.3	17.2	1039.2	23.6
10 mL/min	1017.0	32.1	1084.5	16.1
Neat	890.3	3.7	1062.0	62.0

Table 5: Raw area counts of 1000 ng total fortified samples from variable extraction rates (n=3).

Sample	Nicotine 133 *m/z*	Nicotine (STD)	Methadone 72 *m/z*	Methadone (STD)
1 mL/min	159647.0	21609.1	2399916.7	286448.7
5 mL/min	158923.3	18953.3	2667522.7	79342.9
10 mL/min	158050.3	30493.8	2554780.3	234138.9
Neat	57928.7	26471.0	2981960.0	389256.7

Table 6: Compounds included in method analysis

Compound List		
1 6-monoacetylmorphine	19 Diphenhydramine	37 Nicotine
2 7-aminoclonazepam	20 EDDP	38 Norbuprenorphine
3 Acetaminophen	21 N-Ethcathinone	39 Nordiazepam
4 Alprazolam	22 Fentanyl	40 Norfentanyl
5 Amitriptyline	23 Fluoxetine	41 Norfluoxetine
6 Amphetamine	24 Gabapentin	42 Norketamine
7 Benzoylecgonine	25 Hydrocodone	43 Nortriptyline
8 Buprenorphine	26 Hydromorphone	44 Oxycodone
9 Carisoprodol	27 A-Hydroxyalprazolam	45 Oxymorphone
10 Citalopram	28 Ketamine	46 Paroxetine
11 Clonazepam	29 MDMA	47 Promethazine
12 Cocaethylene	30 Meprobamate	48 Salicylic Acid
13 Cocaine	31 Methadone	49 THC
14 Codeine	32 Methamphetamine	50 THCA-A
15 Cyclobenzaprine	33 Methcathinone	51 Trazodone
16 N-Desmethylcitalopram	34 Midazolam	52 Valproic Acid
17 Dextromethorphan	35 Morphine	53 Zolpidem
18 Diazepam	36 Naloxone	

Table 7: Analyte relative retention times derived from GC/MS run

Drug Name	Retention Time (min)	IS Retention Time (min)	Relative Retention Time
Valproic Acid	5.543	13.853	0.400
Amphetamine	5.970	13.853	0.431
Methamphetamine	6.643	13.853	0.480
Salicylic Acid	7.197	13.853	0.520
n-Propylamphetamine (IS)	7.630	13.850	0.551
Methcathinone	7.687	13.853	0.555
Nicotine	7.740	13.853	0.559
n-ethcathinone	8.203	13.853	0.592
MDMA	9.403	13.853	0.679
Gabapentin	9.703	13.850	0.701
Acetaminophen	10.563	13.850	0.763
Meprobamate	11.490	13.850	0.830
Norketamine	11.727	13.853	0.847
Norfluoxetine	11.807	13.853	0.852
Fluoxetine	11.943	13.850	0.862
Ketamine	11.970	13.850	0.864
Diphenhydramine	12.030	13.850	0.869
Carisoprodol	12.053	13.850	0.870
Norfentanyl	12.693	13.853	0.916
EDDP	13.203	13.850	0.953
Methadone-d9 (IS)	13.850	13.850	1.000
Dextromethorphan	13.863	13.853	1.001
Methadone	13.894	13.850	1.003
Amitriptyline	14.207	13.850	1.026
Cocaine	14.267	13.850	1.030
Nortriptyline	14.323	13.853	1.034
Cyclobenzaprine	14.483	13.850	1.046
Cocaethylene	14.593	13.850	1.054
Promethazine	14.733	13.853	1.064
Codeine	15.370	13.850	1.110
Citalopram	15.413	13.850	1.113
n-desmethylcitalopram	15.570	13.850	1.124
Morphine	15.650	13.853	1.130
Diazepam	15.673	13.850	1.132
Hydrocodone	15.747	13.850	1.137
THCA-A	15.787	13.853	1.140
THC	15.790	13.853	1.140
Hydromorphone	15.820	13.850	1.142
Nordiazepam	16.040	13.853	1.158
6-monoacetylmorphine	16.127	13.850	1.164
Oxycodone	16.190	13.853	1.169
Oxymorphone	16.273	13.853	1.175
Paroxetine	16.367	13.853	1.181
Benzoylecgonine	16.437	13.850	1.187
Midazolam	16.500	13.853	1.191
Naloxone	16.980	13.853	1.226
Fentanyl	17.107	13.850	1.235
Zolpidem	17.563	13.853	1.268
7-aminoclonazepam	17.800	13.853	1.285
Clonazepam	17.843	13.850	1.288
Alprazolam	18.443	13.850	1.332
α-Hydroxyalprazolam	18.987	13.850	1.371
Norbuprenorphine	20.410	13.853	1.473
Trazodone	21.680	13.853	1.565
Buprenorphine	22.517	13.850	1.626

RRT= analyte retention time/IS retention time
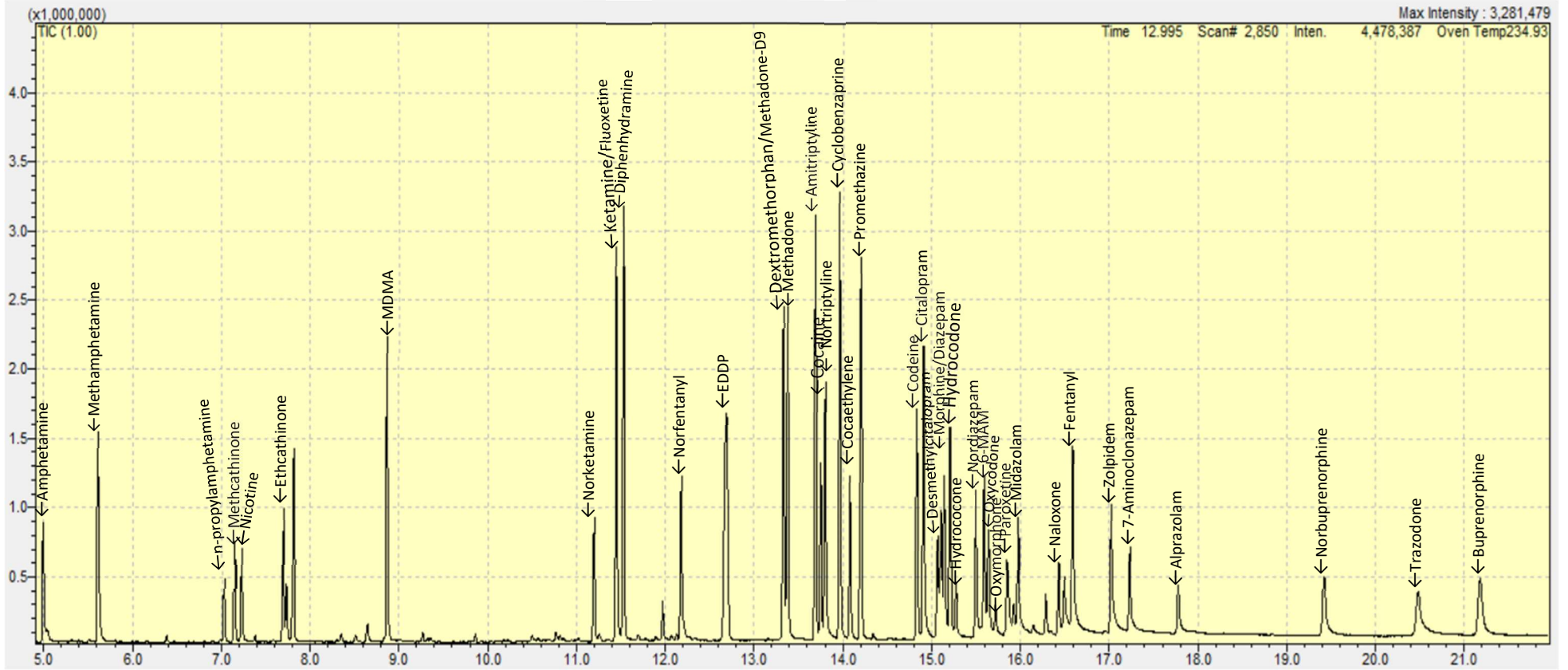
Figure 12: Extracted basic fraction chromatogram with identified compounds labeled
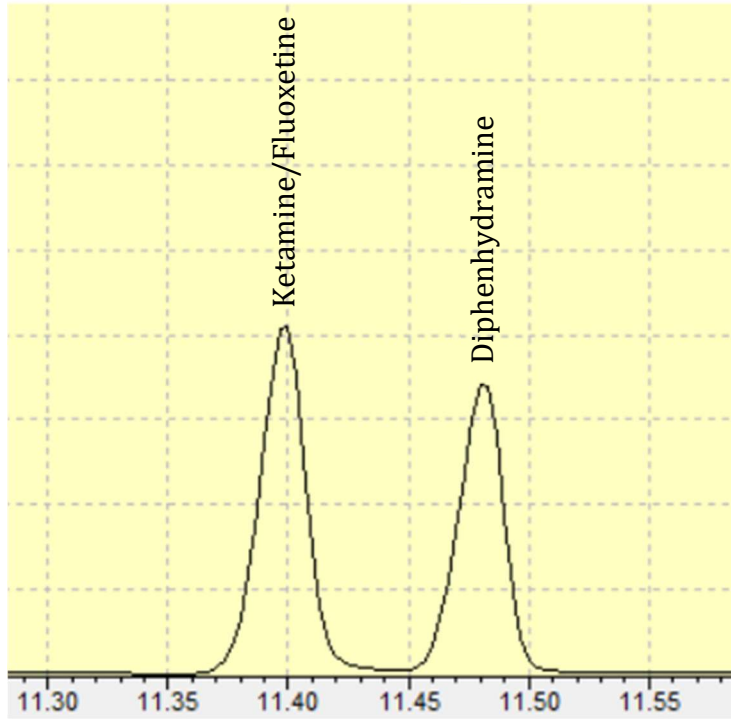
Figure 13: Calibrator full scan chromatogram with overlapping analyte peaks
(Ketamine/Fluoxetine)
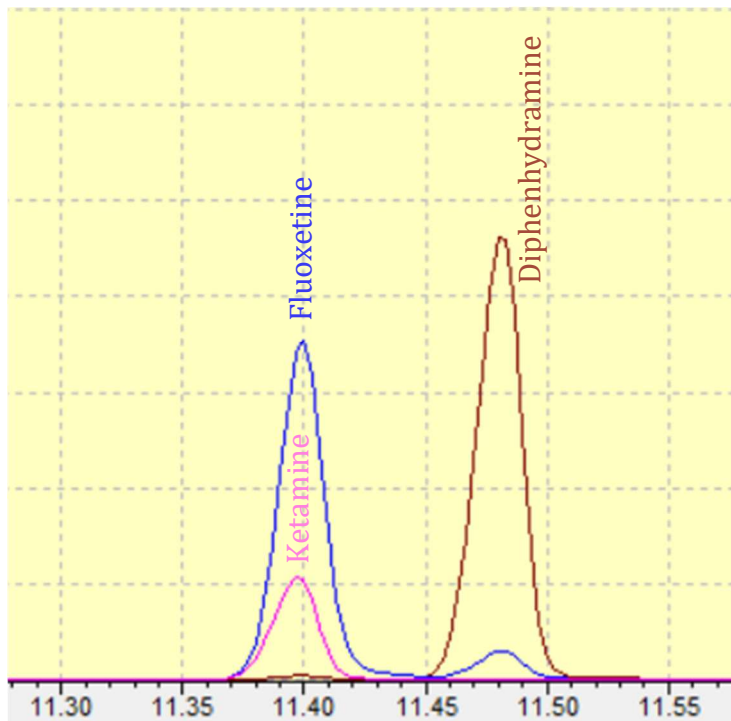
Figure 14: Calibrator extracted ion chromatogram (44, 58, and 180 *m/z*) with overlapping analyte
peaks (Ketamine/Fluoxetine)

Table 8: Quantification ions used for calibration curve construction for each analyte

| Drug Name	Quant Ion (*m/z*)	Drug Name	Quant Ion (*m/z*)
Amphetamine	44	Citalopram	58
Methamphetamine	58	Desmethylcitalopram	44
Methcathinone	58	Morphine	285
Nicotine	84	Diazepam	256
n-Ethcathinone	72	Hydrocodone	299
MDMA	58	Hydromorphone	285
Norketamine	166	Nordiazepam	242
Ketamine	180	6-MAM	327
Fluoxetine	44	Oxycodone	315
Diphenhydramine	58	Oxymorphone	301
Norfentanyl	83	Paroxetine	44
EDDP	277	Benzoylecgonine	124
Dextromethorphan	59	Midazolam	310
Methadone-D9	78	Naloxone	327
Methadone	72	Fentanyl	245
Amitriptyline	58	Zolpidem	235
Cocaine	82	7-aminoclonazepam	285
Nortriptyline	44	Alprazolam	279
Cyclobenzaprine	58	Norbuprenorphine	338
Cocaethylene	82	Trazodone	205
Promethazine	72	Buprenorphine	378
Codeine	299		
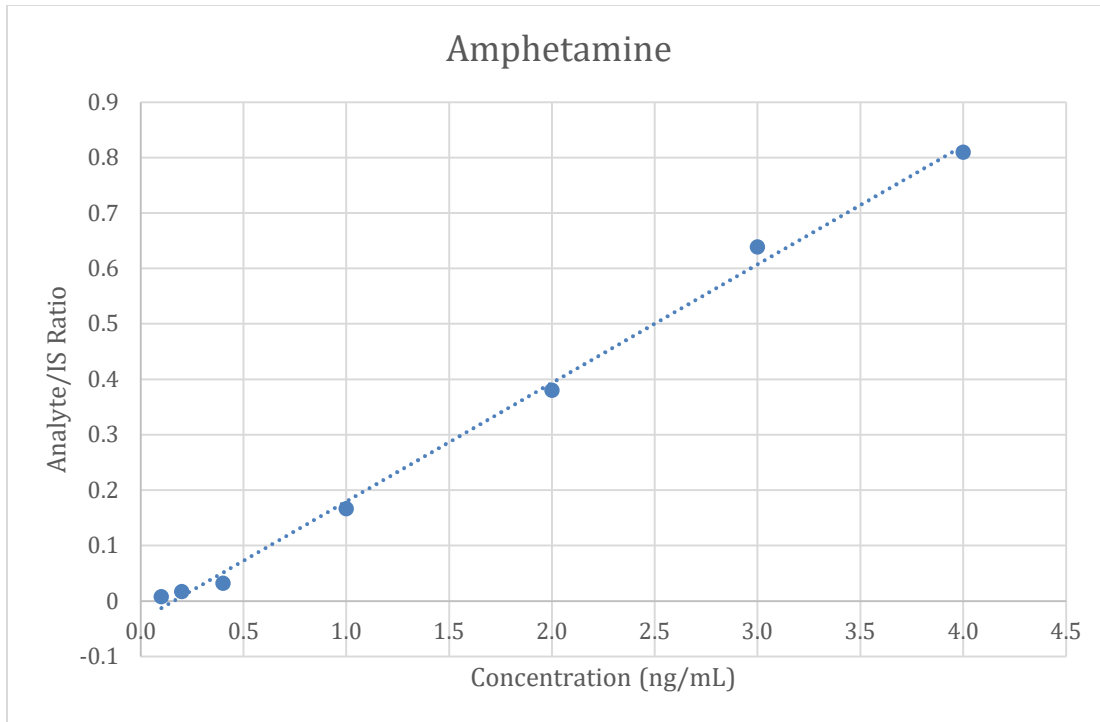
Figure 15: Amphetamine calibration curve run 03/16/2020. $R^2 = 0.9963$

Table 9: Bias for amphetamine calibration curve run 03/16/2020.

Amphetamine		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.19	94.1
0.20	0.24	18.4
0.40	0.31	-23.2
1.00	0.94	-6.4
2.00	1.94	-3.2
3.00	3.15	4.9
4.00	3.94	-1.4
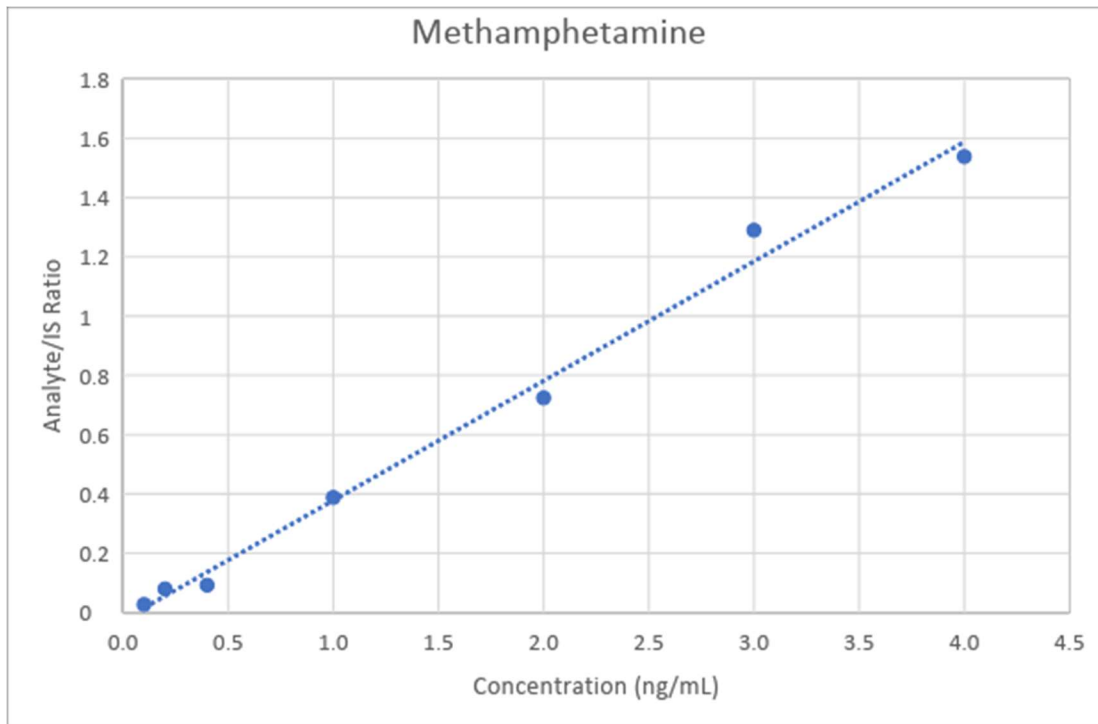
Figure 16: Methamphetamine calibration curve run 03/16/2020. $R^2 = 0.9914$

Table 10: Bias for methamphetamine calibration curve run 03/16/2020.

| | Methamphetamine | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.13	29.1
0.20	0.26	30.0
0.40	0.29	-27.3
1.00	1.02	2.5
2.00	1.86	-7.1
3.00	3.26	8.7
4.00	3.88	-3.1
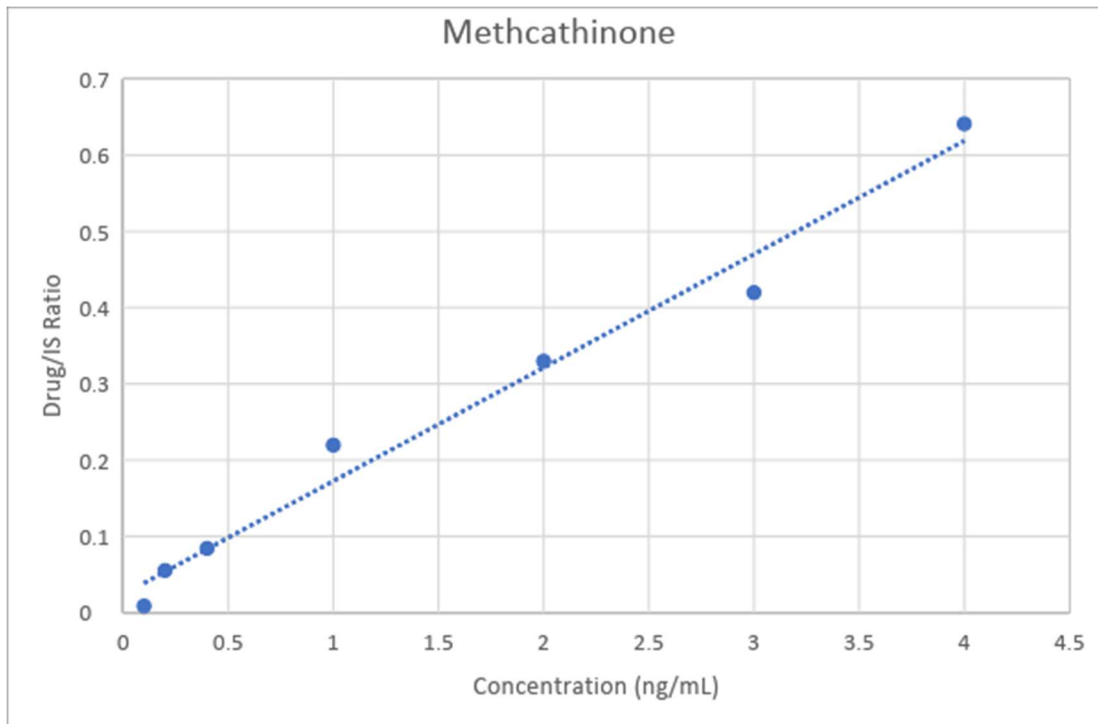
Figure 17: Methcathinone calibration curve run 03/16/2020. R^2= 0.9801

Table 11: Bias for methcathinone calibration curve run 03/16/2020.

| | Methcathinone | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	-0.10	-201.7
0.20	0.21	5.8
0.40	0.41	1.5
1.00	1.32	31.7
2.00	2.06	2.8
3.00	2.66	-11.3
4.00	4.15	3.7
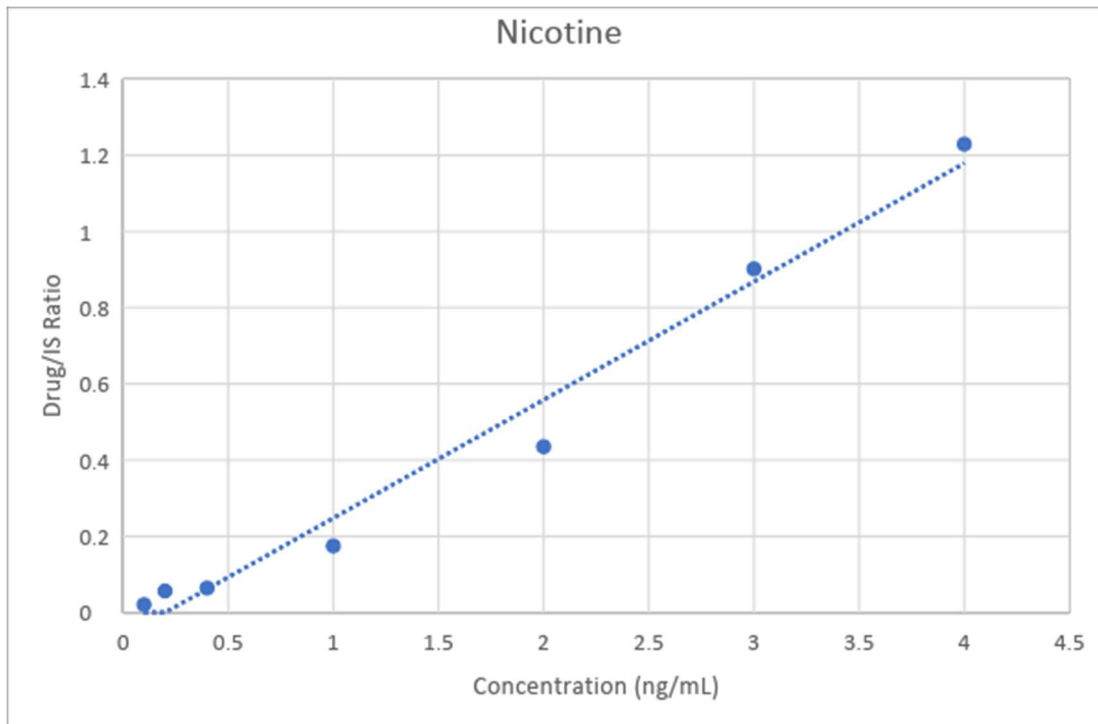
Figure 18: Nicotine calibration curve run 03/16/2020. $R^2 = 0.9780$

Table 11: Bias for nicotine calibration curve run 03/16/2020.

	Nicotine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.27	168.9
0.20	0.38	92.1
0.40	0.41	2.2
1.00	0.76	-23.5
2.00	1.60	-19.8
3.00	3.11	3.6
4.00	4.16	4.1
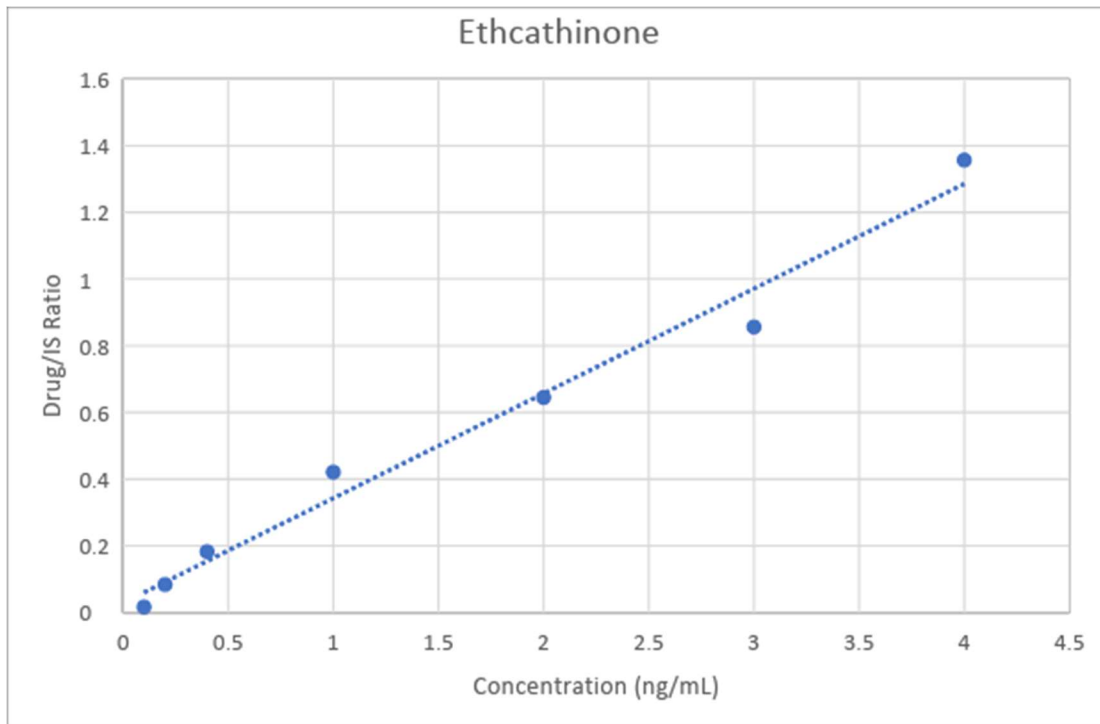
Figure 19: Ethcathinone calibration curve run 03/16/2020. $R^2 = 0.9805$

Table 12: Bias for ethcathinone calibration curve run 03/16/2020.

| | Ethcathinone | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	-0.04	-138.1
0.20	0.18	-11.9
0.40	0.49	22.5
1.00	1.25	24.9
2.00	1.96	-1.9
3.00	2.63	-12.2
4.00	4.23	5.7
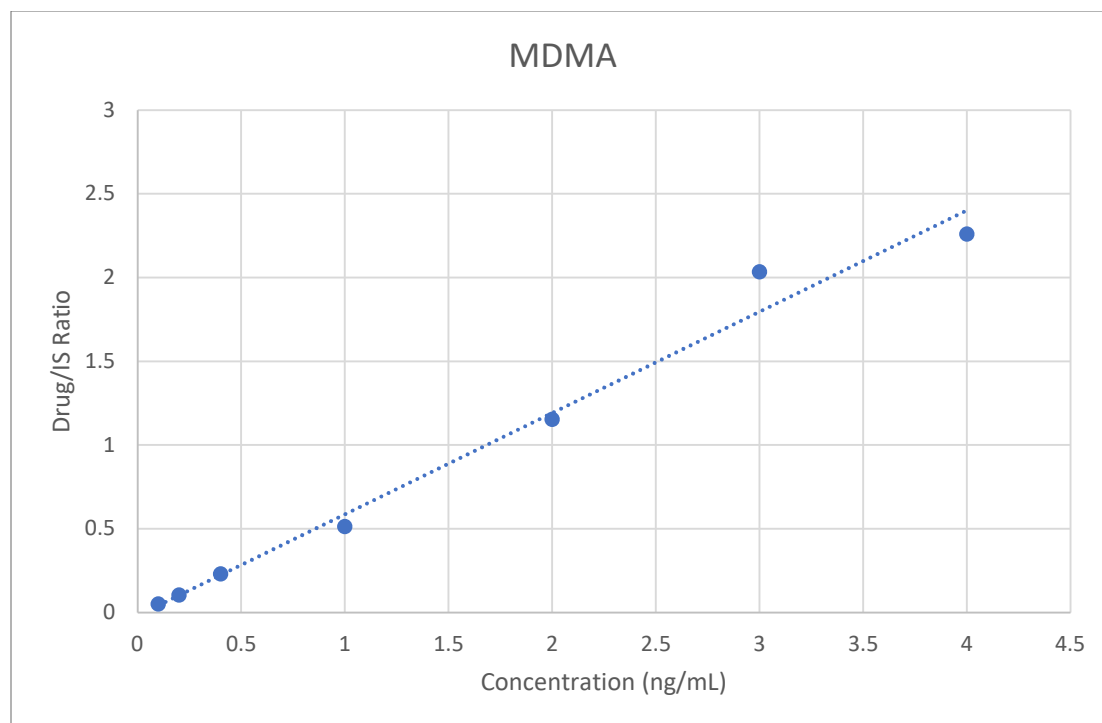
Figure 20: 3,4-methylenedioxymethamphetamine calibration curve run 03/16/2020. $R^2 = 0.9838$

Table 13: Bias for 3,4-methylenedioxymethamphetamine calibration curve run 03/16/2020.

MDMA		
Theoretical Conc. (ng/mL)	**Calc. Conc. (ng/mL)**	**Bias (%)**
0.10	0.06	-35.3
0.20	0.17	-17.1
0.40	0.41	2.0
1.00	0.95	-5.1
2.00	2.18	8.8
3.00	3.86	28.8
4.00	4.30	7.4
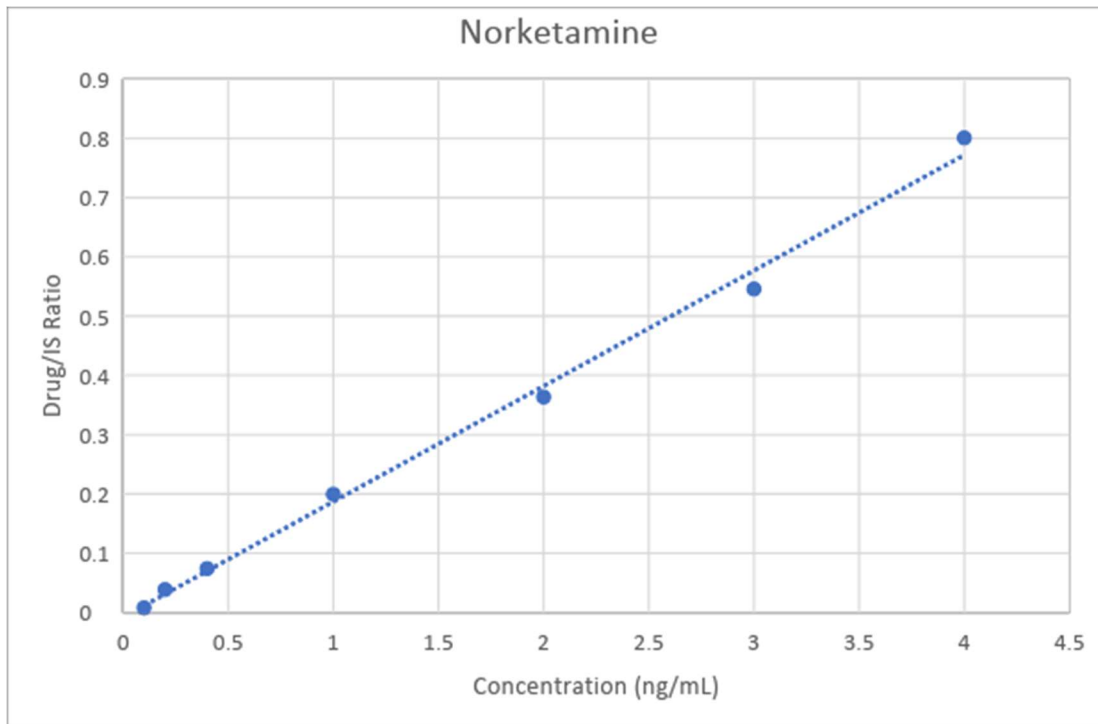
Figure 21: Norketamine calibration curve run 03/16/2020. $R^2 = 0.9955$

Table 14: Bias for norketamine calibration curve run 03/16/2020.

	Norketamine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.08	-17.7
0.20	0.24	19.9
0.40	0.42	5.3
1.00	1.06	6.4
2.00	1.91	-4.7
3.00	2.84	-5.3
4.00	4.15	3.7
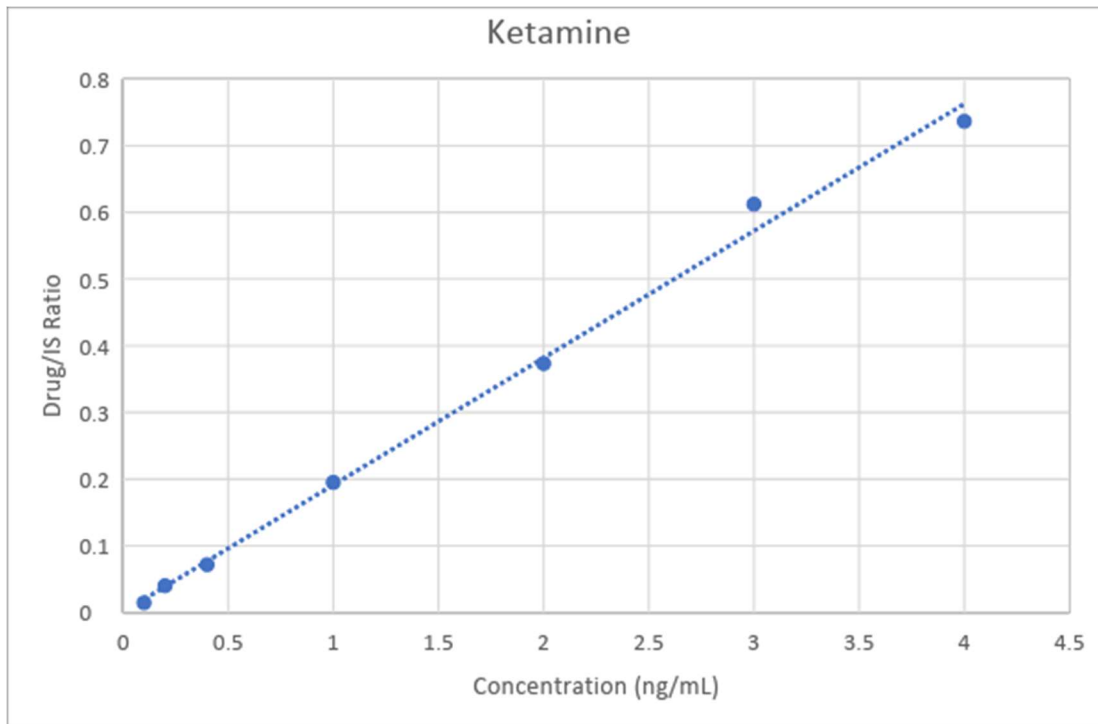
Figure 22: Ketamine calibration curve run 03/16/2020. $R^2 = 0.9952$

Table 14: Bias for ketamine calibration curve run 03/16/2020.

	Ketamine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.07	-27.9
0.20	0.21	3.2
0.40	0.37	-6.9
1.00	1.02	2.0
2.00	1.96	-2.2
3.00	3.21	7.0
4.00	3.86	-3.4
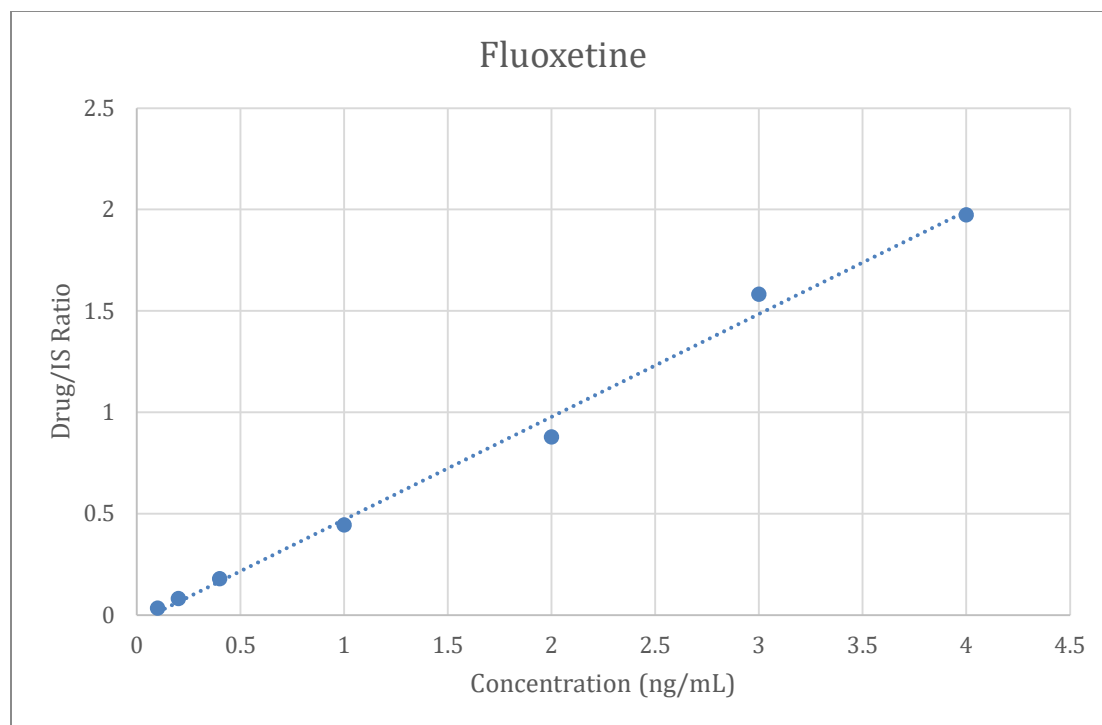
Figure 23: Fluoxetine calibration curve run 03/16/2020. $R^2= 0.9941$

Table 15: Bias for fluoxetine calibration curve run 03/16/2020.

Fluoxetine		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.14	39.0
0.20	0.23	15.8
0.40	0.42	6.2
1.00	0.95	-5.3
2.00	1.80	-9.8
3.00	3.19	6.4
4.00	3.96	-0.9
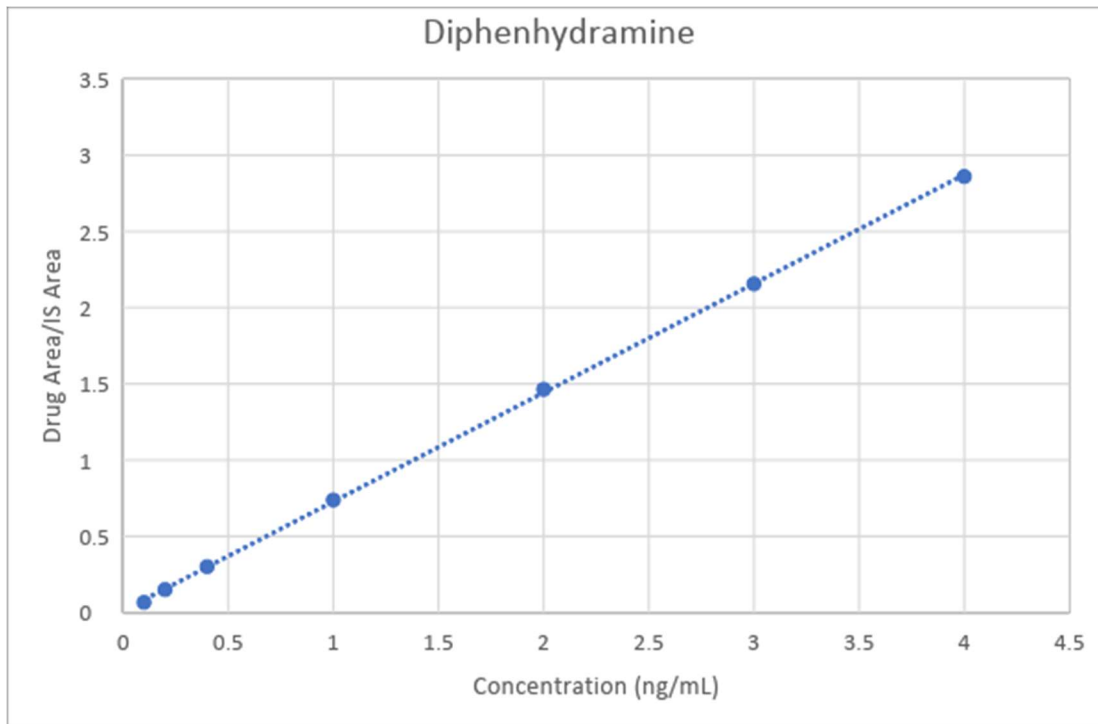
Figure 24: Diphenhydramine calibration curve run 03/16/2020. R^2= 0.9999

Table 16: Bias for diphenhydramine calibration curve run 03/16/2020.

Theoretical Conc. (ng/mL)	Diphenhydramine Calc. Conc. (ng/mL)	Bias (%)
0.10	0.08	-23.9
0.20	0.19	-3.3
0.40	0.40	1.1
1.00	1.01	1.5
2.00	2.03	1.5
3.00	3.00	-0.1
4.00	3.98	-0.4
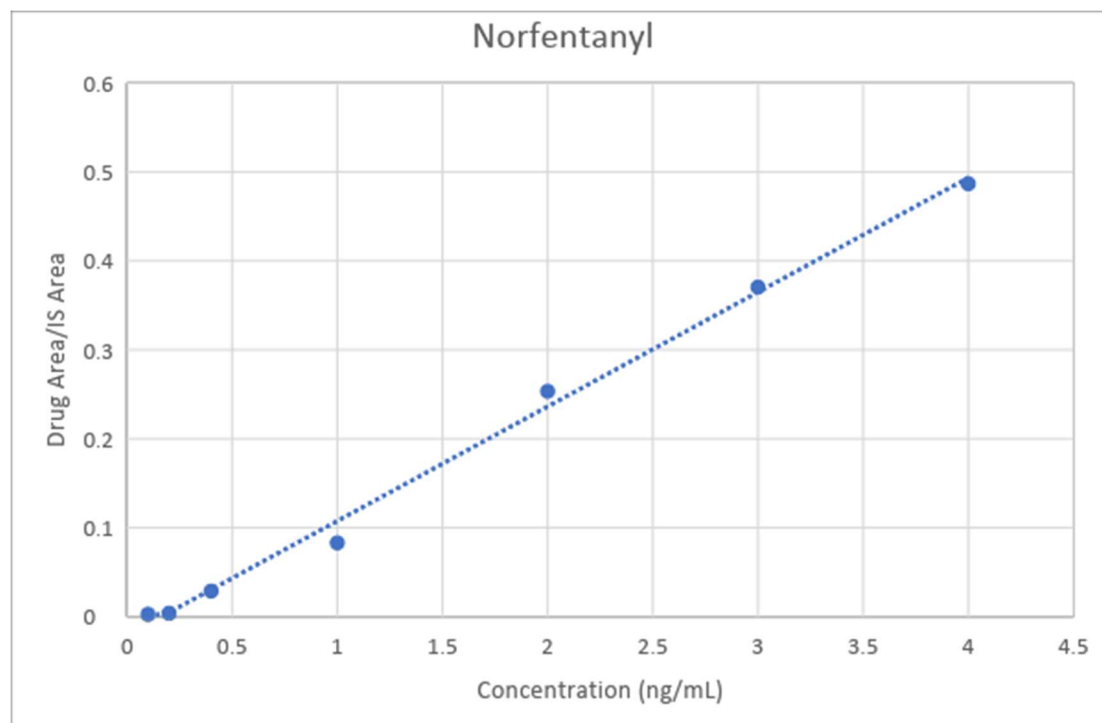
Figure 25: Norfentanyl calibration curve run 03/16/2020. $R^2 = 0.9952$

Table 17: Bias for norfentanyl calibration curve run 03/16/2020.

	Norfentanyl	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.1	0.18	84.9
0.2	0.19	-3.1
0.4	0.39	-3.3
1	0.81	-19.2
2	2.13	6.6
3	3.04	1.4
4	3.95	-1.3
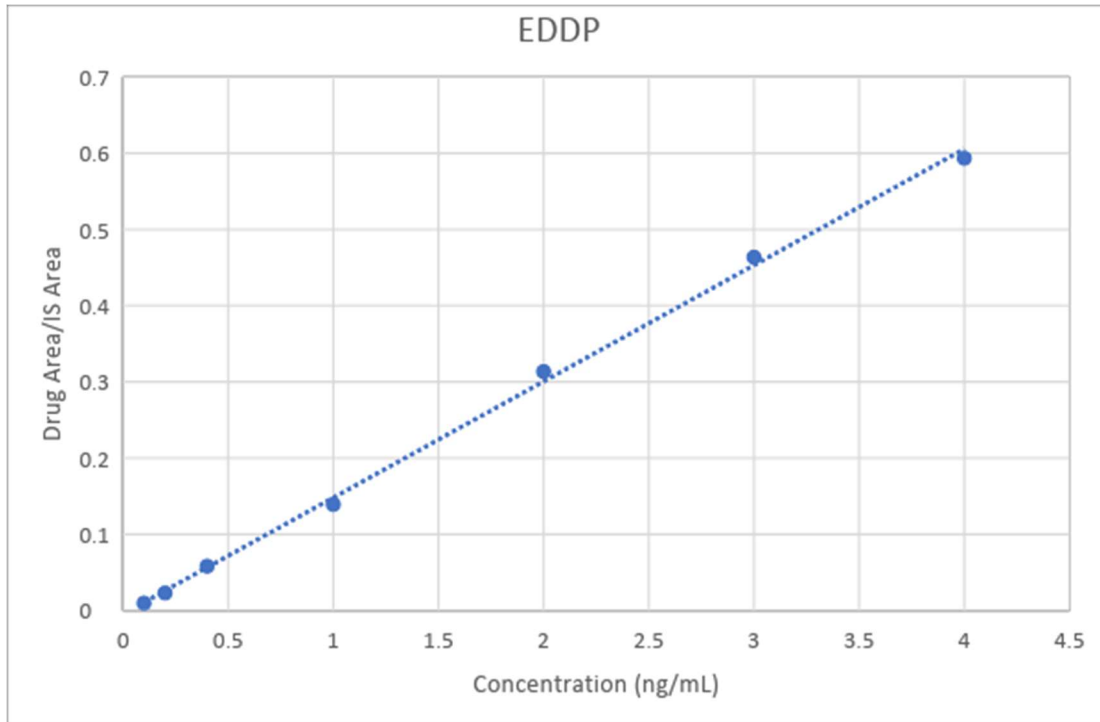
Figure 26: EDDP calibration curve run 03/16/2020. R^2= 0.9984

Table 18: Bias for EDDP calibration curve run 03/16/2020.

	EDDP	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.09	-7.0
0.20	0.18	-10.0
0.40	0.41	2.7
1.00	0.94	-5.7
2.00	2.09	4.3
3.00	3.07	2.3
4.00	3.92	-2.0
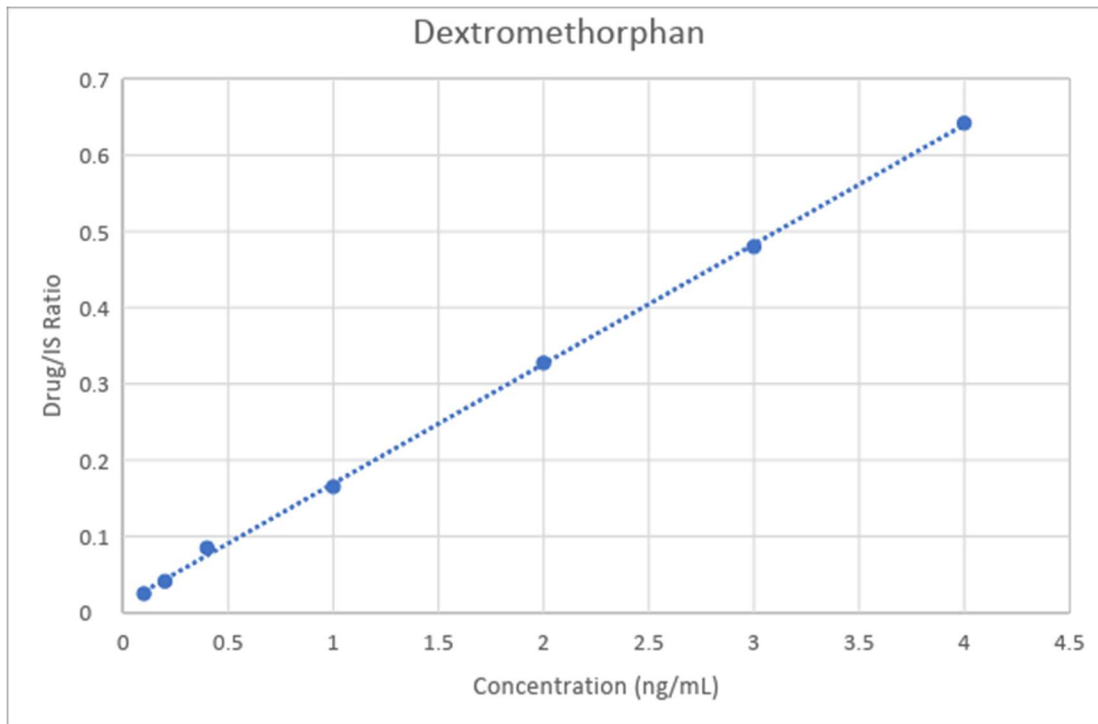
Figure 27: Dextromethorphan calibration curve run 03/16/2020. R^2= 0.9996

Table 19: Bias for dextromethorphan calibration curve run 03/16/2020.

| | Dextromethorphan | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.08	-19.9
0.20	0.18	-8.2
0.40	0.46	15.5
1.00	0.97	-2.6
2.00	2.01	0.4
3.00	2.98	-0.7
4.00	4.01	0.3
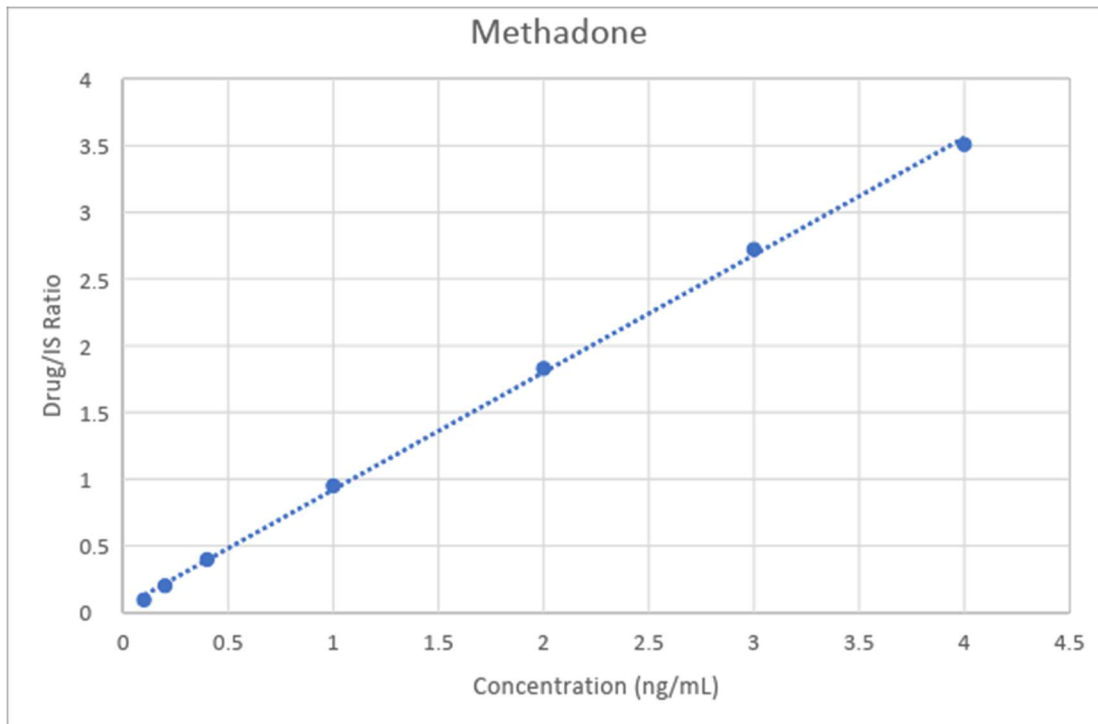
Figure 28: Methadone calibration curve run 03/16/2020. R^2= 0.9993

Table 20: Bias for methadone calibration curve run 03/16/2020.

	Methadone	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.06	-39.2
0.20	0.18	-9.5
0.40	0.40	1.1
1.00	1.03	3.2
2.00	2.03	1.6
3.00	3.05	1.6
4.00	3.94	-1.5
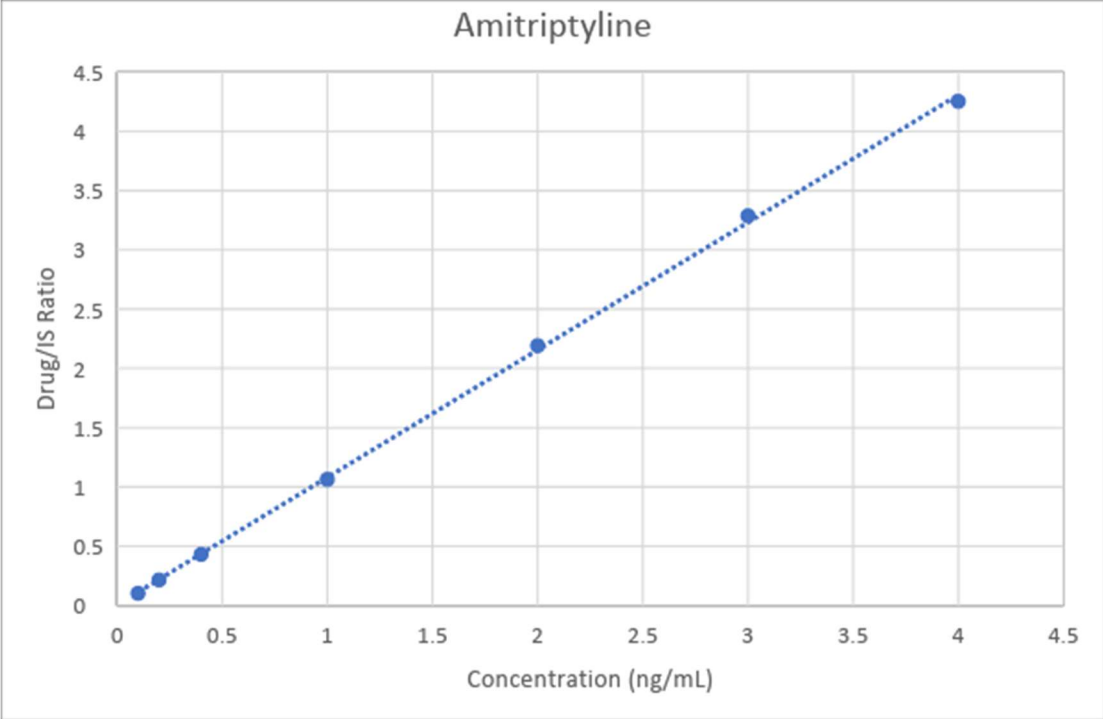
Figure 29: Amitriptyline calibration curve run 03/16/2020. $R^2 = 0.9995$

Table 21: Bias for amitriptyline calibration curve run 03/16/2020.

|	Amitriptyline	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.09	-9.9
0.20	0.20	-2.2
0.40	0.40	-1.1
1.00	0.98	-1.6
2.00	2.03	1.7
3.00	3.05	1.7
4.00	3.95	-1.3
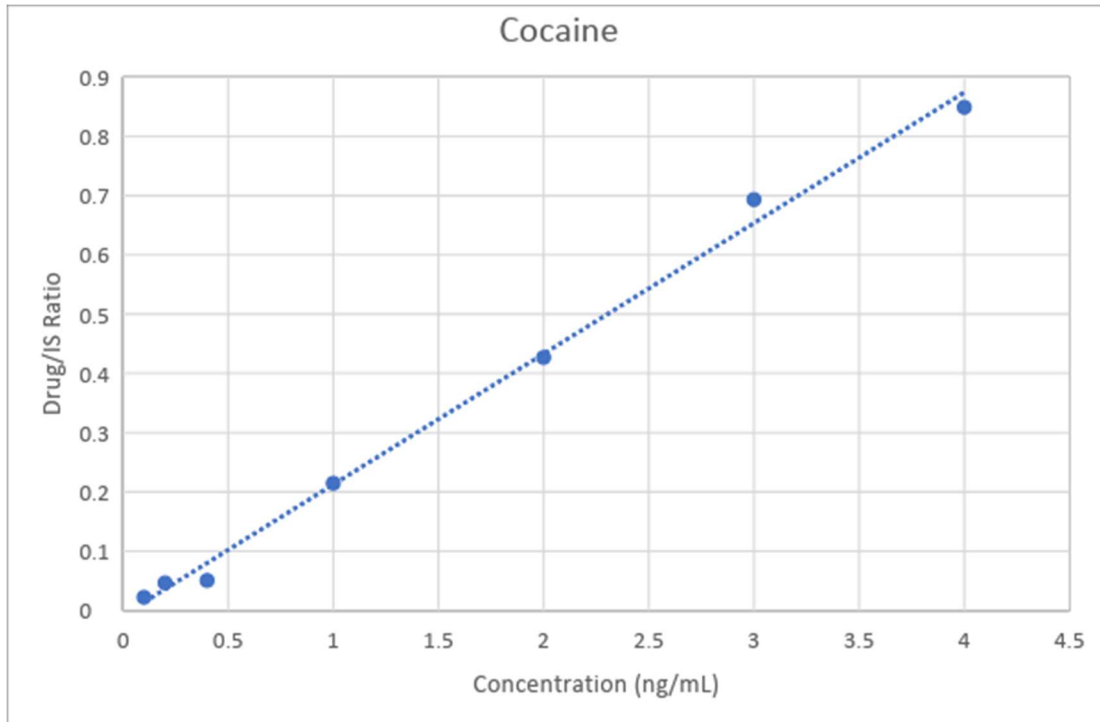
Figure 30: Cocaine calibration curve run 03/16/2020. $R^2= 0.9951$

Table 22: Bias for cocaine calibration curve run 03/16/2020.

| | Cocaine | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.14	37.5
0.20	0.25	23.9
0.40	0.27	-33.4
1.00	1.01	1.0
2.00	1.97	-1.4
3.00	3.18	6.0
4.00	3.89	-2.8
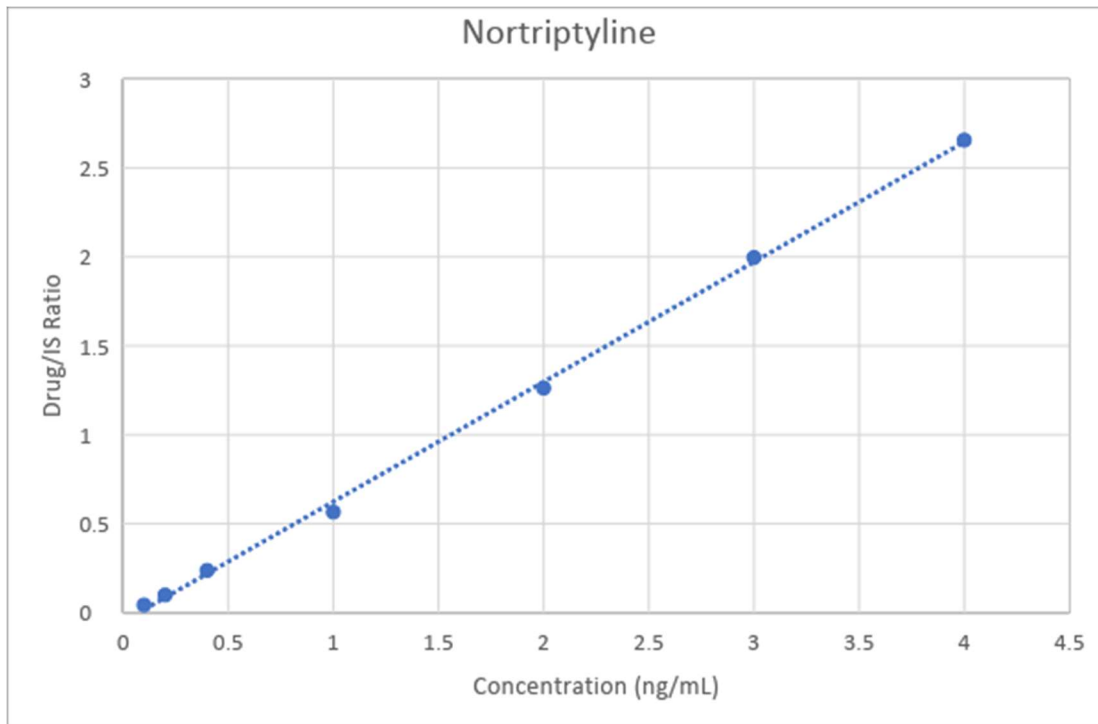
Figure 31: Nortriptyline calibration curve run 03/16/2020. $R^2 = 0.9990$

Table 23: Bias for nortriptyline calibration curve run 03/16/2020.

| | Nortriptyline | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.14	38.4
0.20	0.22	10.7
0.40	0.43	6.7
1.00	0.91	-8.6
2.00	1.95	-2.6
3.00	3.04	1.2
4.00	4.02	0.4
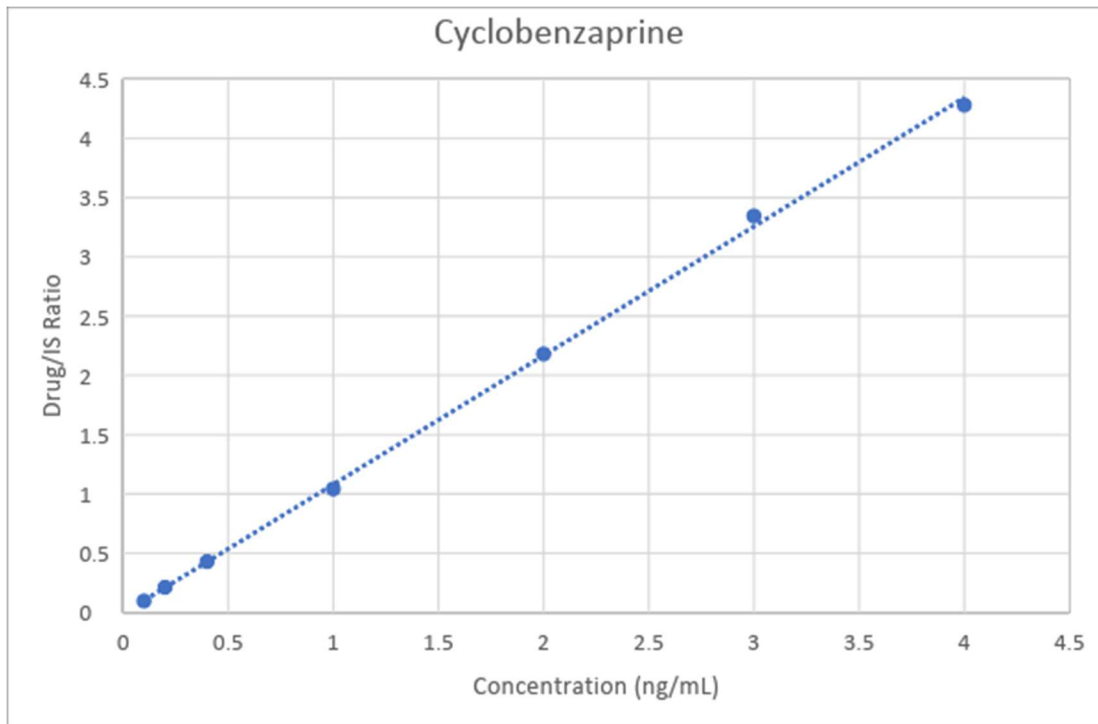
Figure 32: Cyclobenzaprine calibration curve run 03/16/2020. $R^2 = 0.9992$

Table 24: Bias for cyclobenzaprine calibration curve run 03/16/2020.

| | Cyclobenzaprine | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.10	-3.9
0.20	0.20	1.2
0.40	0.40	0.5
1.00	0.97	-3.5
2.00	2.01	0.6
3.00	3.08	2.7
4.00	3.94	-1.5
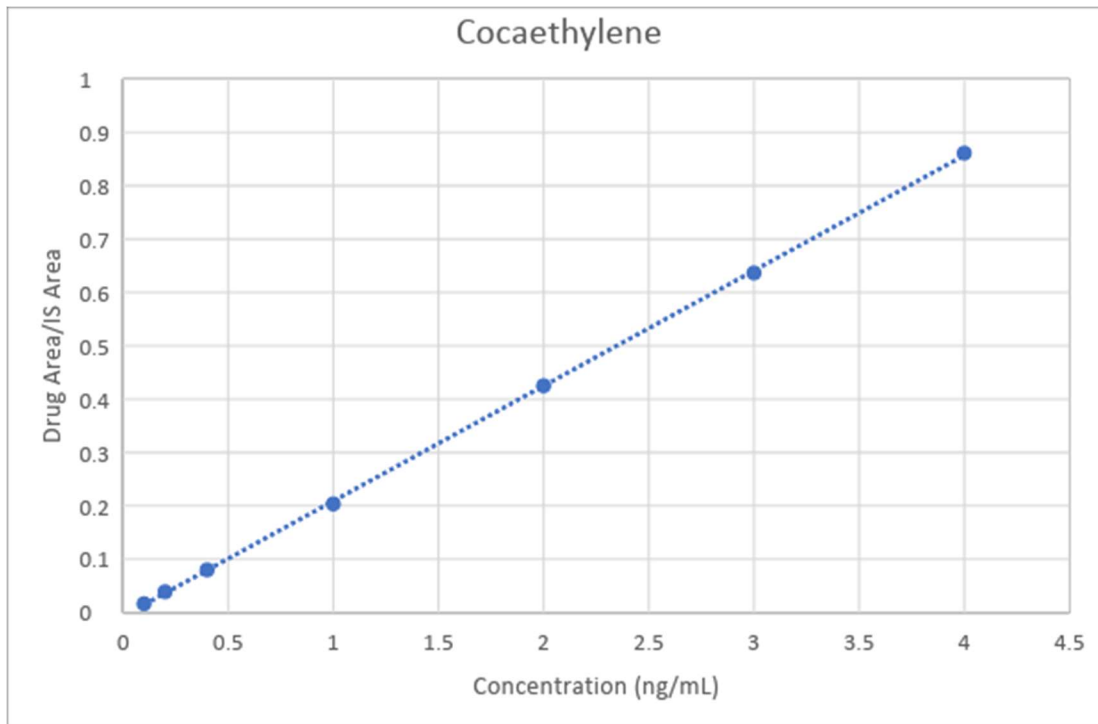
Figure 33: Cocaethylene calibration curve run 03/16/2020. $R^2 = 0.9999$

Table 25: Bias for cocaethylene calibration curve run 03/16/2020.

	Cocaethylene	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.11	8.5
0.20	0.21	6.2
0.40	0.40	0.7
1.00	0.98	-2.5
2.00	2.00	0.0
3.00	2.98	-0.7
4.00	4.02	0.5
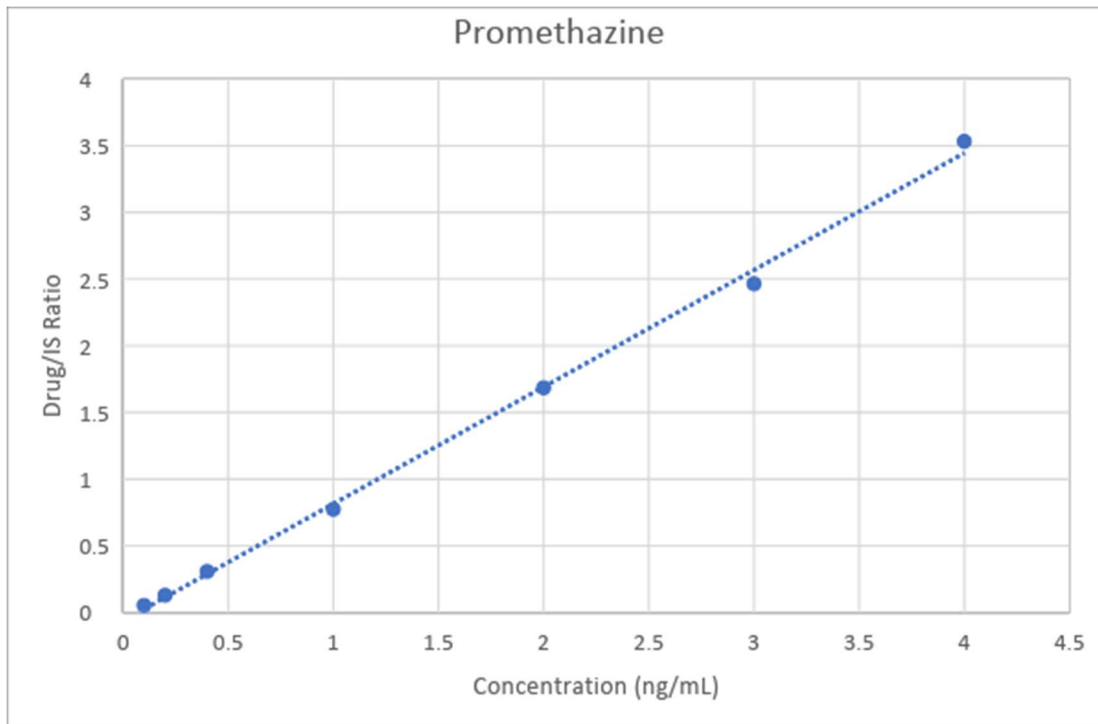
Figure 34: Promethazine calibration curve run 03/16/2020. R^2= 0.9980

Table 26: Bias for promethazine calibration curve run 03/16/2020.

	Promethazine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.13	31.9
0.20	0.22	9.3
0.40	0.42	5.4
1.00	0.95	-4.7
2.00	1.99	-0.4
3.00	2.88	-3.9
4.00	4.10	2.5
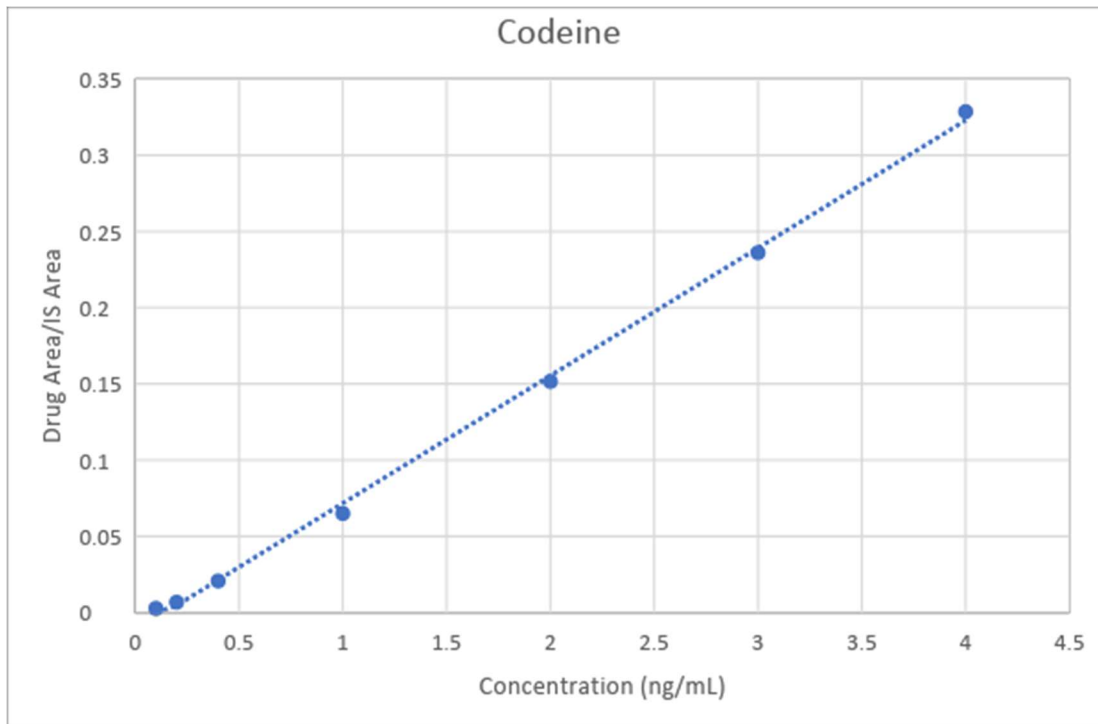
Figure 35: Codeine calibration curve run 03/16/2020. $R^2= 0.9985$

Table 27: Bias for codeine calibration curve run 03/16/2020.

	Codeine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.18	77.3
0.20	0.23	12.7
0.40	0.39	-2.0
1.00	0.92	-7.9
2.00	1.96	-2.2
3.00	2.96	-1.2
4.00	4.07	1.8
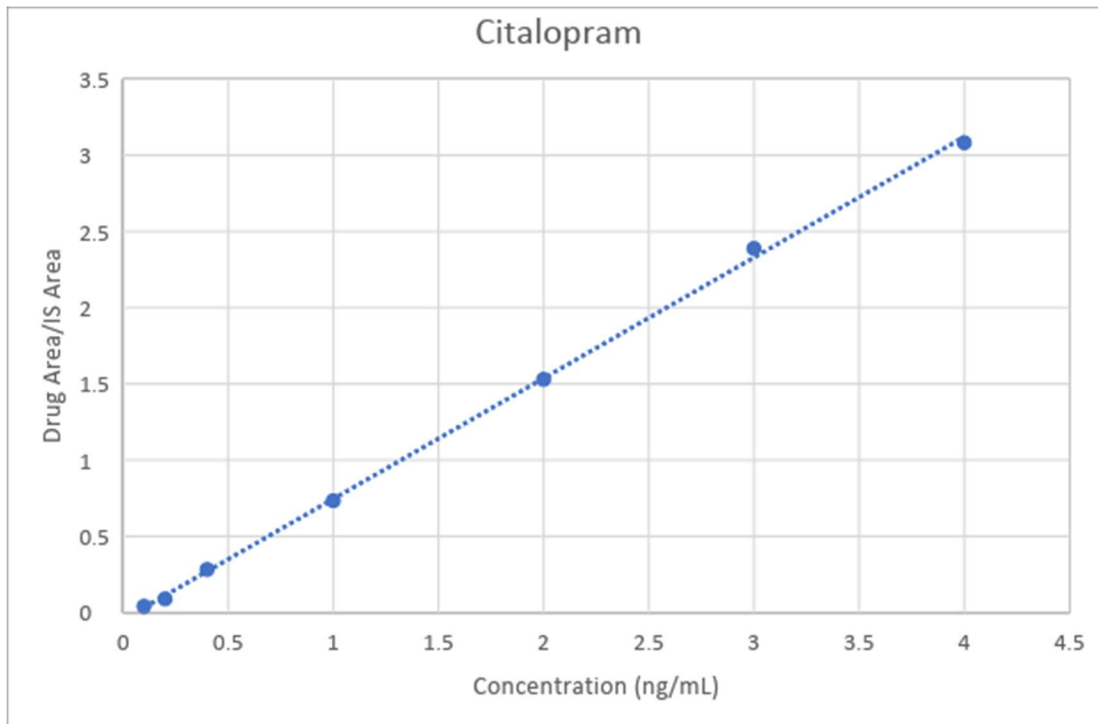
Figure 36: Citalopram calibration curve run 03/16/2020. $R^2 = 0.9993$

Table 28: Bias for citalopram calibration curve run 03/16/2020.

	Citalopram	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.11	10.6
0.20	0.17	-14.2
0.40	0.42	3.8
1.00	0.99	-1.5
2.00	1.99	-0.5
3.00	3.08	2.5
4.00	3.95	-1.2
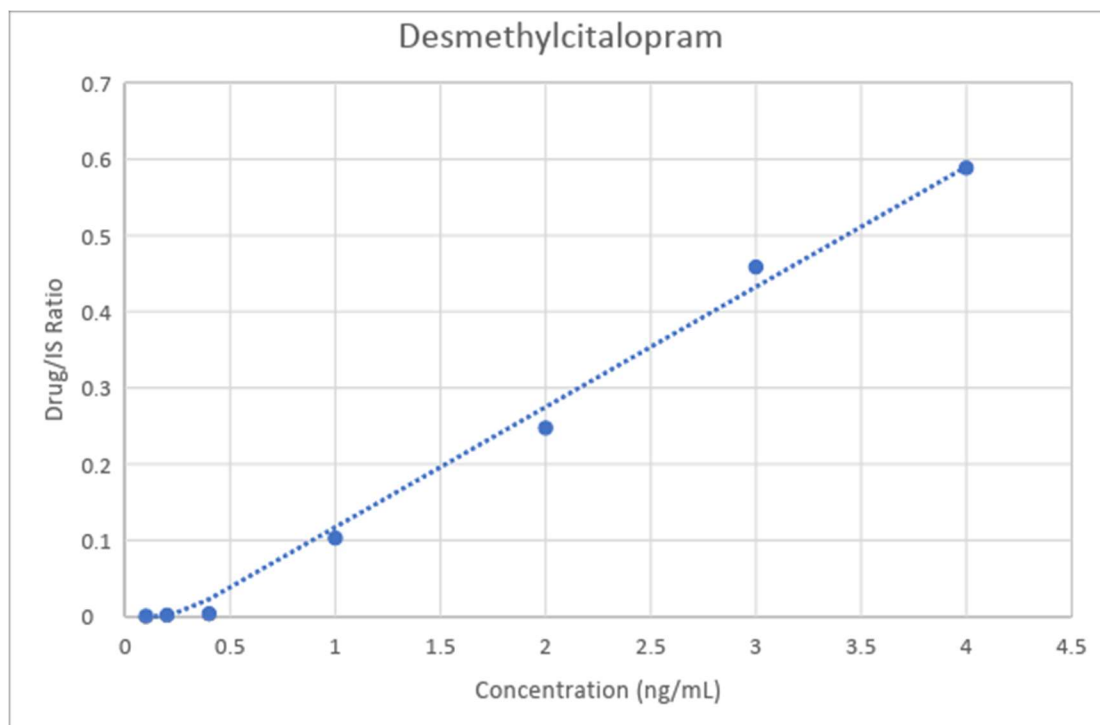
Figure 37: Desmethylcitalopram calibration curve run 03/16/2020. $R^2 = 0.9921$

Table 29: Bias for desmethylcitalopram calibration curve run 03/16/2020.

	Desmethylcitalopram	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.26	160.3
0.20	0.27	34.1
0.40	0.28	-29.9
1.00	0.91	-9.0
2.00	1.83	-8.7
3.00	3.17	5.5
4.00	3.99	-0.2
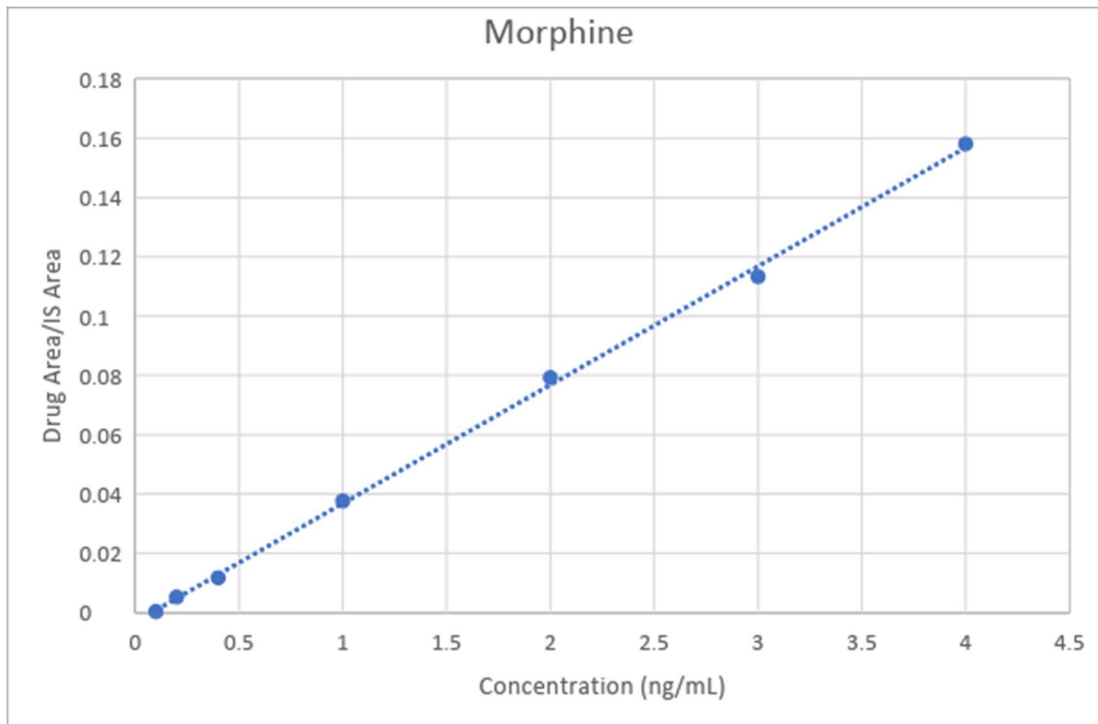
Figure 28: Morphine calibration curve run 03/16/2020. $R^2= 0.9990$

Table 30: Bias for morphine calibration curve run 03/16/2020.

| | Morphine | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.09	-11.3
0.20	0.21	5.4
0.40	0.37	-6.7
1.00	1.02	2.1
2.00	2.06	3.1
3.00	2.91	-2.9
4.00	4.03	0.8
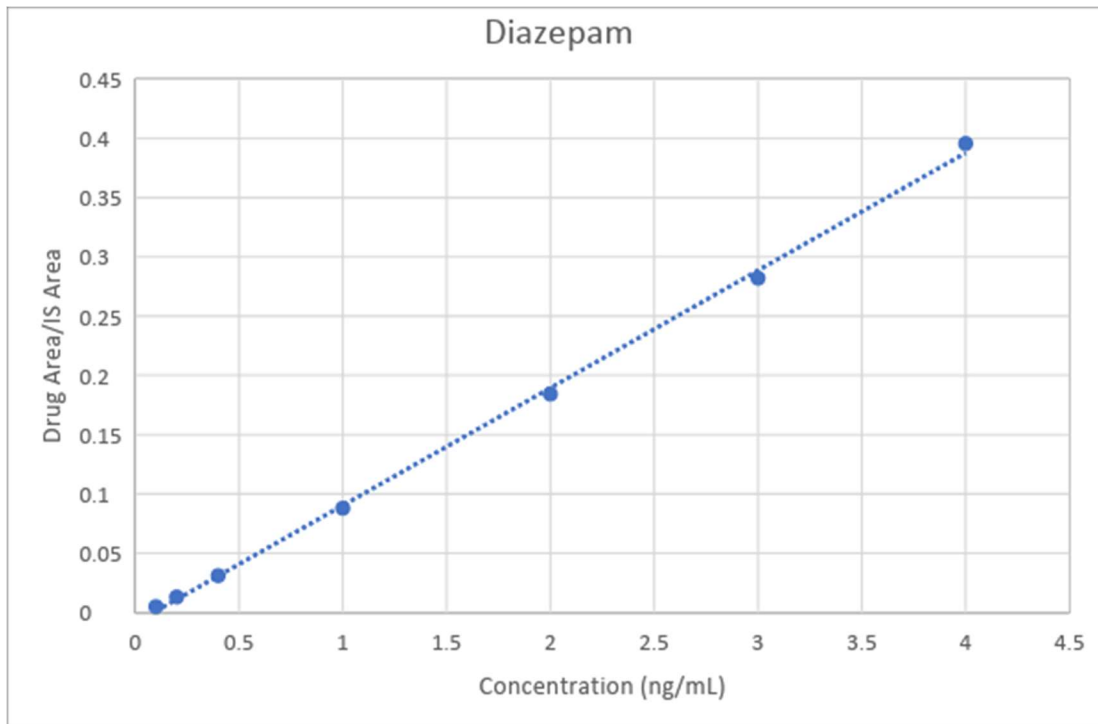
Figure 39: Diazepam calibration curve run 03/16/2020. R^2= 0.9988

Table 31: Bias for diazepam calibration curve run 03/16/2020.

| | Diazepam | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.14	39.1
0.20	0.22	10.6
0.40	0.40	0.7
1.00	0.98	-2.4
2.00	1.95	-2.6
3.00	2.93	-2.2
4.00	4.08	2.0
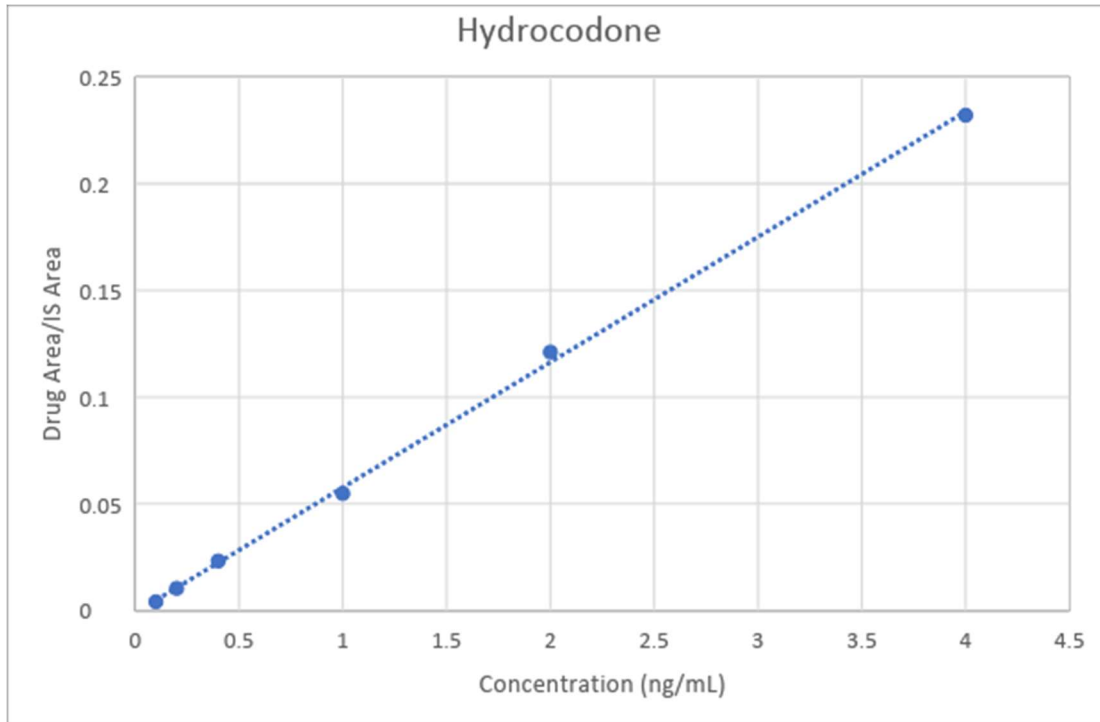
Figure 40: Hydrocodone calibration (3.0 ng/mL point dropped due to being an outlier) curve run 03/16/2020. R^2= 0.9991

Table 32: Bias for hydrocodone calibration curve run 03/16/2020.

	Hydrocodone	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.09	-11.2
0.20	0.20	-2.2
0.40	0.41	3.5
1.00	0.95	-4.7
2.00	2.08	4.1
~~3.00~~	~~1.99~~	~~-33.5~~
4.00	3.97	-0.7
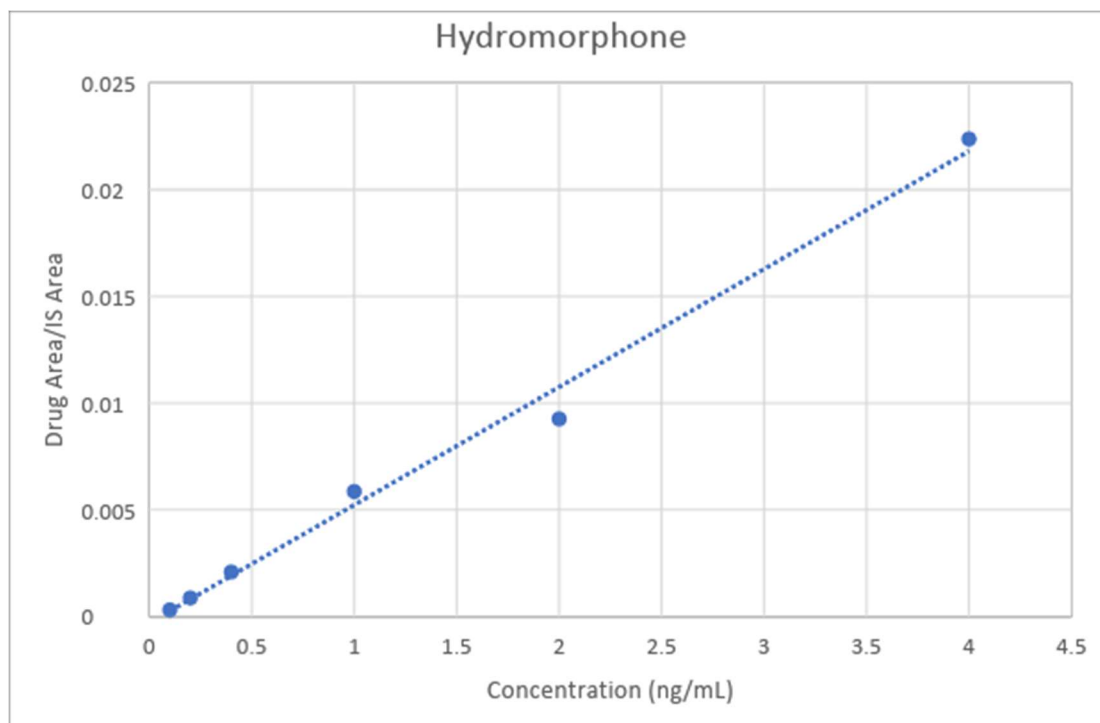
Figure 41: Hydromorphone calibration (3.0 ng/mL point dropped due to being an outlier) curve run 03/16/2020. $R^2 = 0.9914$

Table 33: Bias for hydromorphone calibration curve run 03/16/2020.

	Hydromorphone	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.11	11.0
0.20	0.21	6.5
0.40	0.43	8.6
1.00	1.12	12.0
2.00	1.74	-13.1
3.00	2.03	-32.4
4.00	4.12	3.1
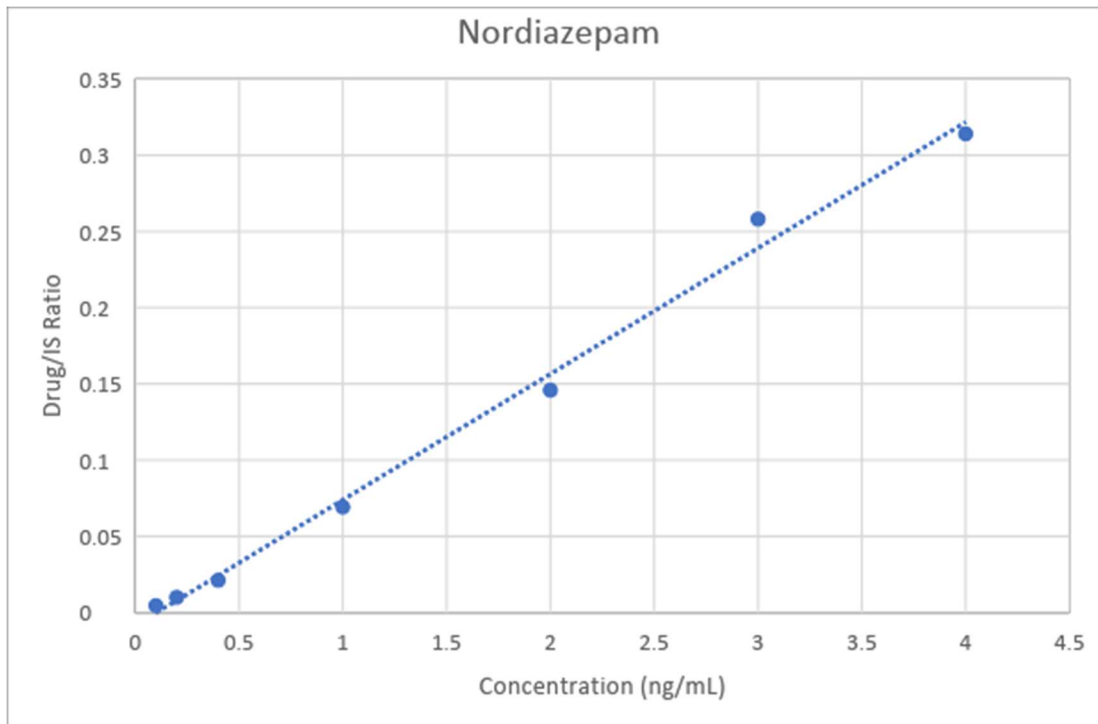
Figure 42: Nordiazepam calibration curve run 03/16/2020. $R^2= 0.9938$

Table 34: Bias for nordiazepam calibration curve run 03/16/2020.

| | Nordiazepam | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.16	60.4
0.20	0.23	13.0
0.40	0.36	-9.8
1.00	0.94	-5.7
2.00	1.87	-6.4
3.00	3.23	7.6
4.00	3.91	-2.3
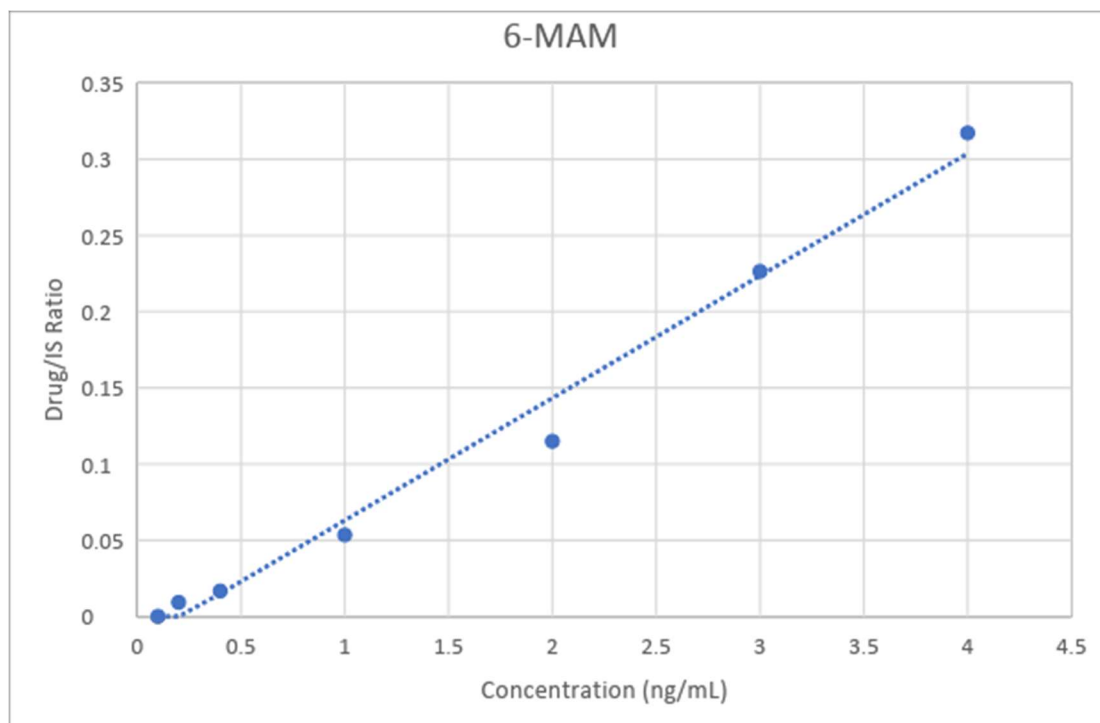
Figure 43: 6-monoacetylmorphine calibration curve run 03/16/2020. $R^2 = 0.9858$

Table 35: Bias for 6-monoacetylmorphine calibration curve run 03/16/2020.

	6-monoacetylmorphine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.22	116.5
0.20	0.33	65.2
0.40	0.42	5.8
1.00	0.88	-11.8
2.00	1.65	-17.6
3.00	3.04	1.2
4.00	4.17	4.2
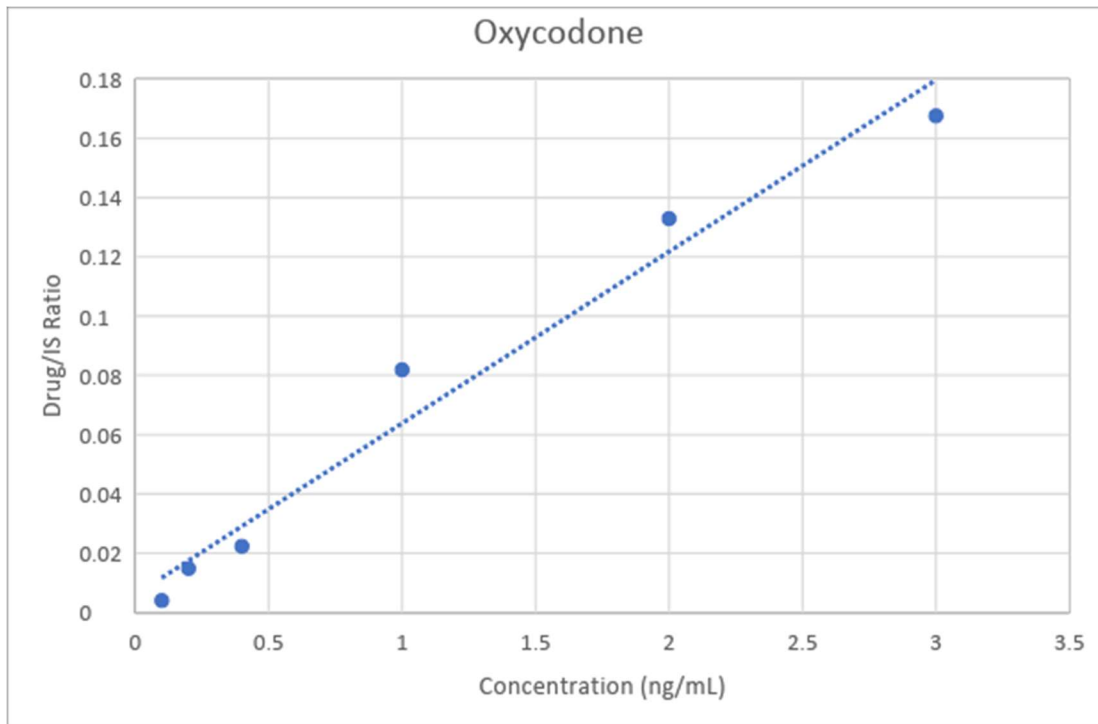
Figure 44: Oxycodone calibration curve run 03/16/2020. R^2= 0.9696

Table 36: Bias for oxycodone calibration curve run 03/16/2020.

| | Oxycodone | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	-0.03	-131.9
0.20	0.15	-22.6
0.40	0.28	-29.0
1.00	1.31	31.3
2.00	2.19	9.7
3.00	2.79	-6.9
4.00	6.99	74.7
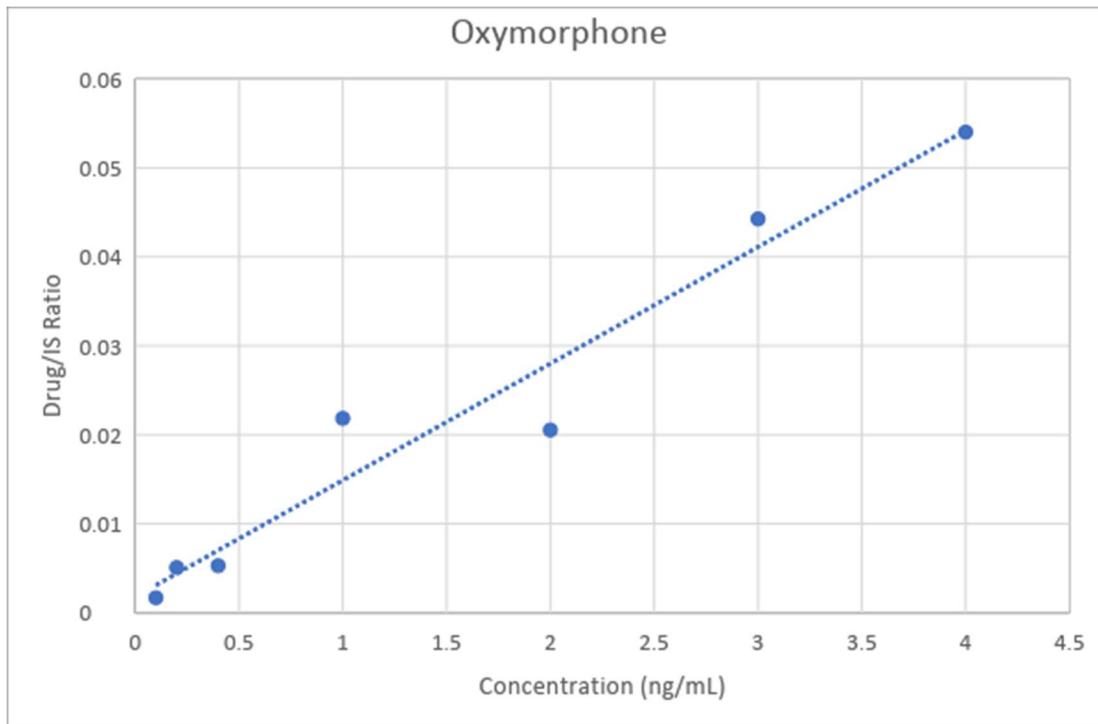
Figure 45: Oxymorphone calibration curve run 03/16/2020. R^2= 0.9522

Table 37: Bias for oxymorphone calibration curve run 03/16/2020.

| | Oxymorphone | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	-0.01	-110.3
0.20	0.25	24.4
0.40	0.26	-33.9
1.00	1.53	53.0
2.00	1.43	-28.5
3.00	3.24	8.0
4.00	3.99	-0.3
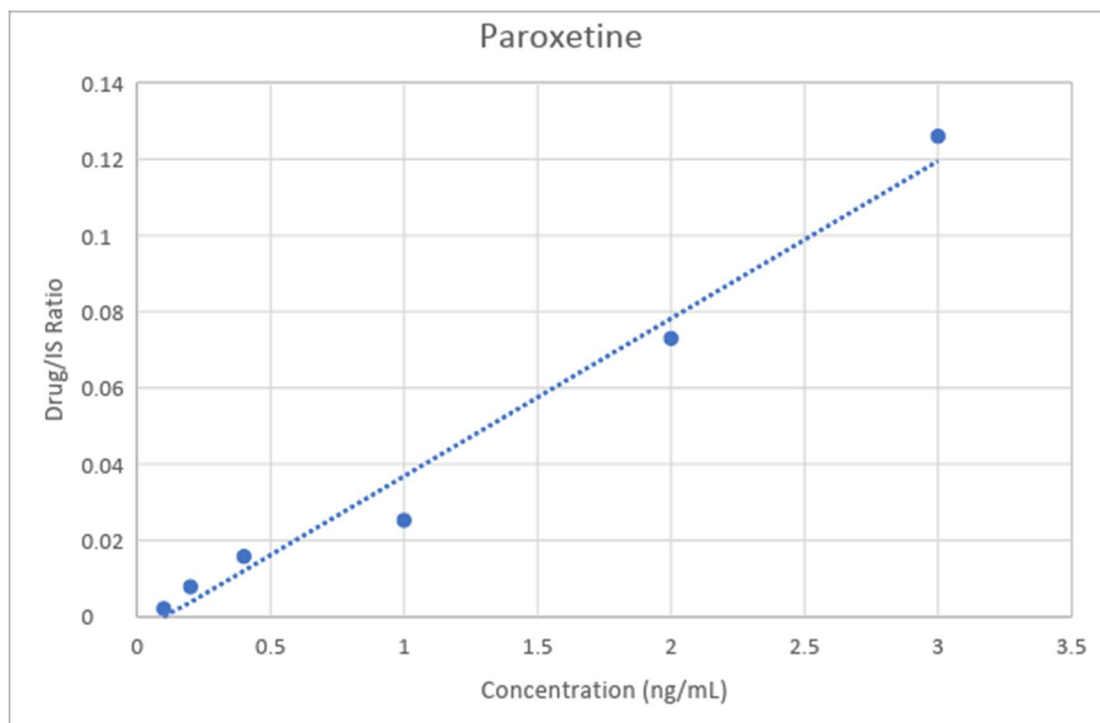
Figure 46: Paroxetine calibration curve run 03/16/2020. $R^2 = 0.9796$

Table 38: Bias for paroxetine calibration curve run 03/16/2020.

	Paroxetine	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.16	58.7
0.20	0.30	48.6
0.40	0.49	22.4
1.00	0.72	-28.1
2.00	1.87	-6.4
3.00	3.15	5.1
4.00	2.42	-39.4
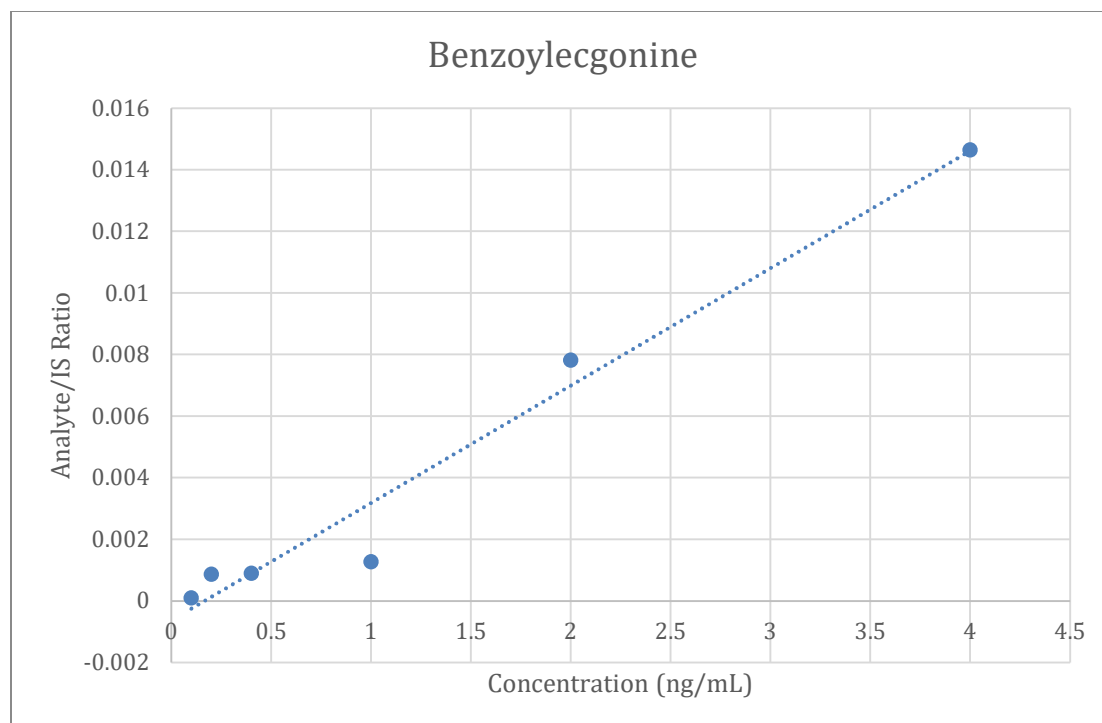
Figure 47: Benzoylecgonine calibration (3.0 ng/mL point dropped due to being an outlier) curve run 03/16/2020. $R^2= 0.9705$

Table 39: Bias for benzoylecgonine calibration curve run 03/16/2020.

| Benzoylecgonine | | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.18	82.8
0.20	0.38	92.1
0.40	0.39	-1.4
1.00	0.49	-50.9
2.00	2.21	10.7
3.00	6.06	102.1
4.00	4.01	0.3
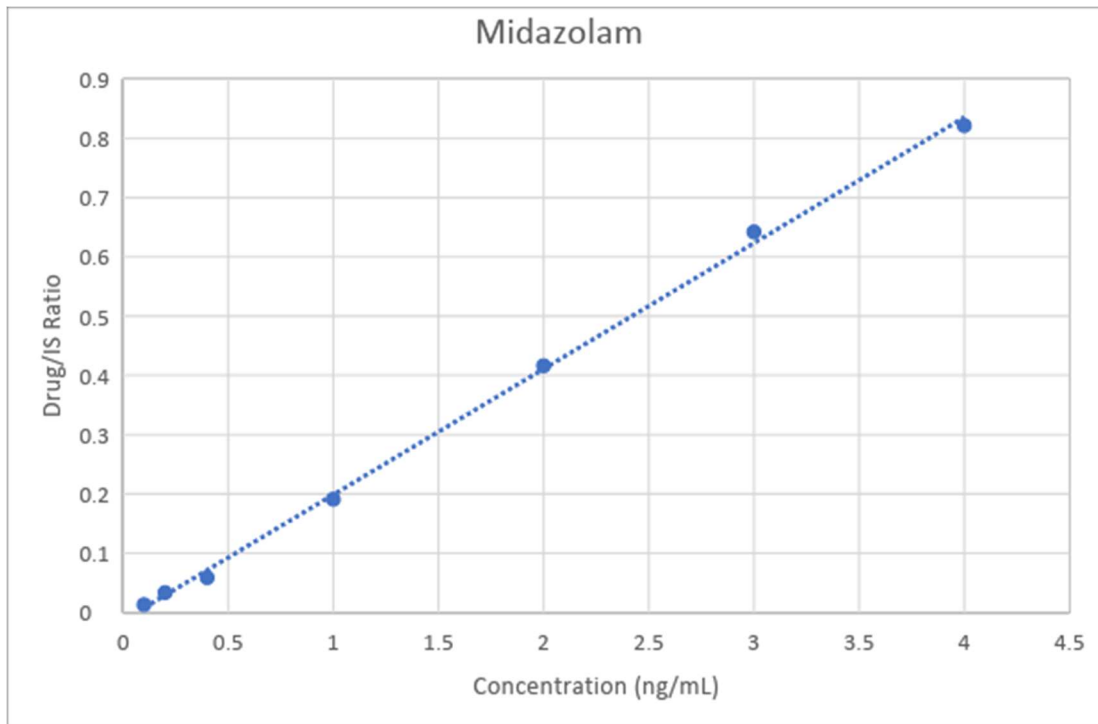
Figure 48: Midazolam calibration curve run 03/16/2020. $R^2 = 0.9986$

Table 40: Bias for midazolam calibration curve run 03/16/2020.

Midazolam		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.13	25.5
0.20	0.22	10.8
0.40	0.34	-14.8
1.00	0.96	-3.6
2.00	2.02	1.2
3.00	3.09	3.0
4.00	3.93	-1.6
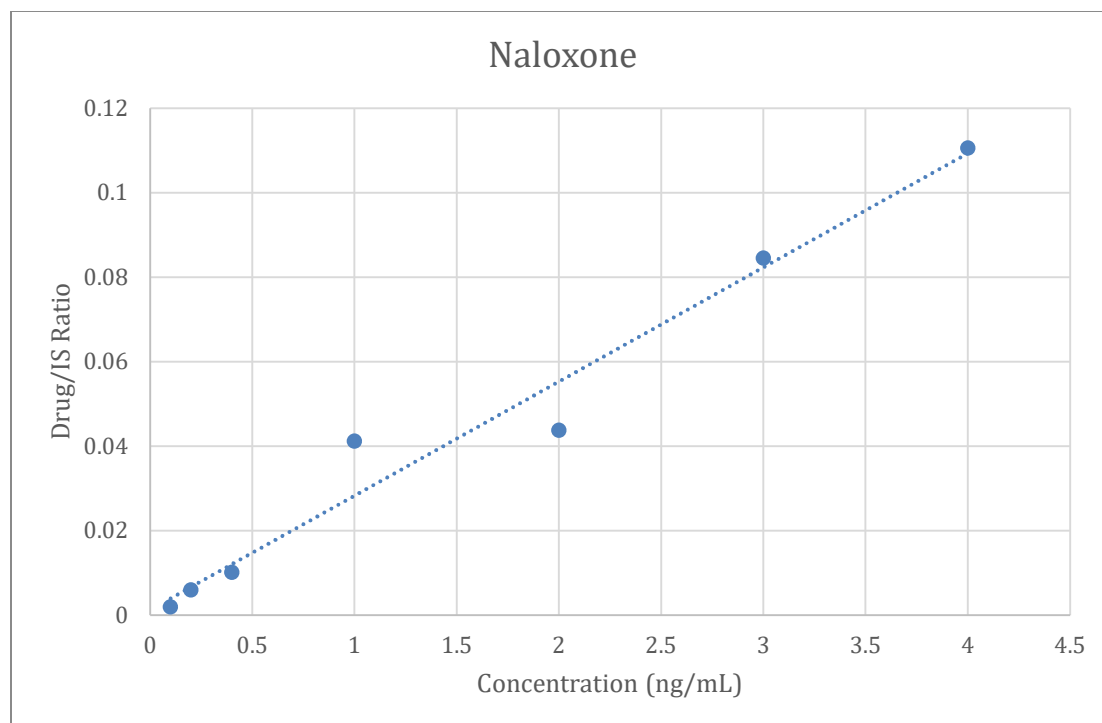
Figure 49: Naloxone calibration run 03/16/2020. $R^2= 0.9698$

Table 41: Bias for naloxone calibration curve run 03/16/2020.

	Naloxone	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.03	-74.9
0.20	0.17	-13.5
0.40	0.33	-18.4
1.00	1.48	47.7
2.00	1.57	-21.4
3.00	3.08	2.7
4.00	4.05	1.2
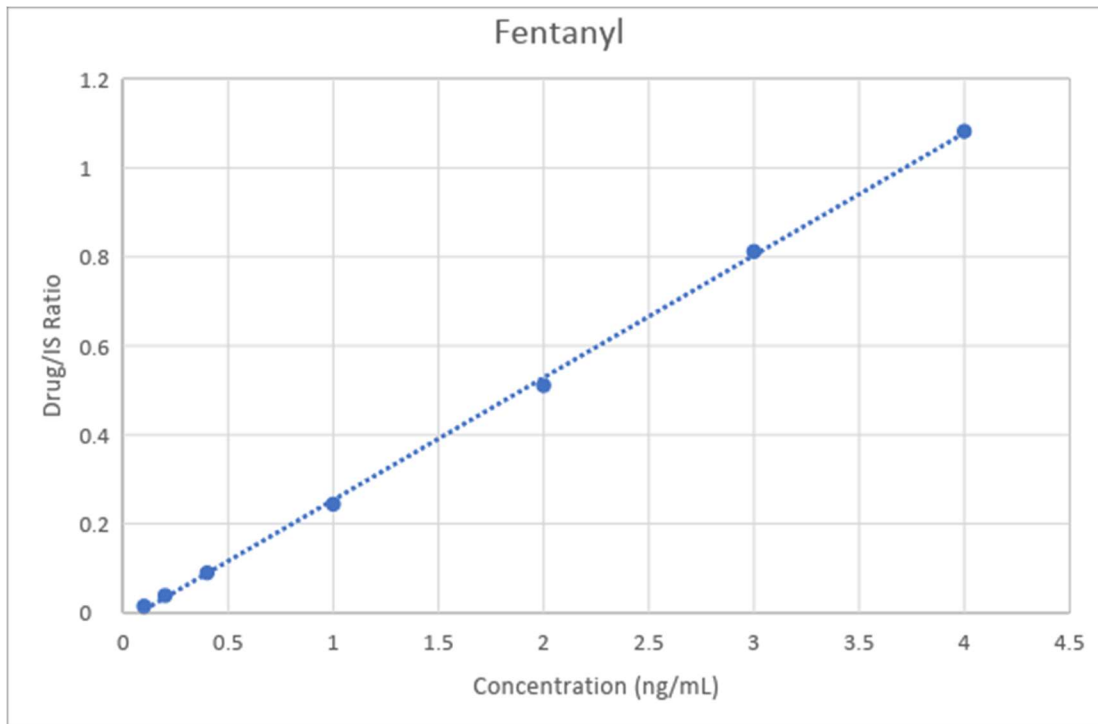
Figure 50: Fentanyl calibration curve run 03/16/2020. $R^2 = 0.9994$

Table 42: Bias for fentanyl calibration curve run 03/16/2020.

	Fentanyl	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.13	30.9
0.20	0.22	9.0
0.40	0.40	0.8
1.00	0.97	-3.5
2.00	1.94	-3.2
3.00	3.03	1.1
4.00	4.01	0.4
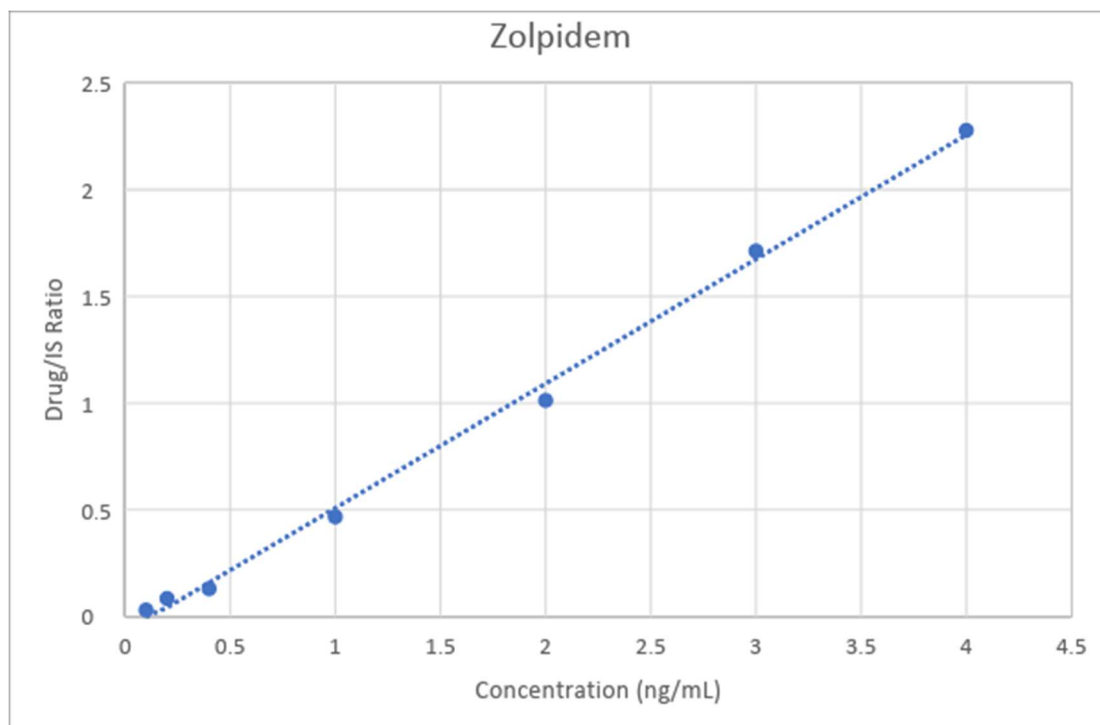
Figure 51: Zolpidem calibration curve run 03/16/2020. $R^2 = 0.9969$

Table 43: Bias for zolpidem calibration curve run 03/16/2020.

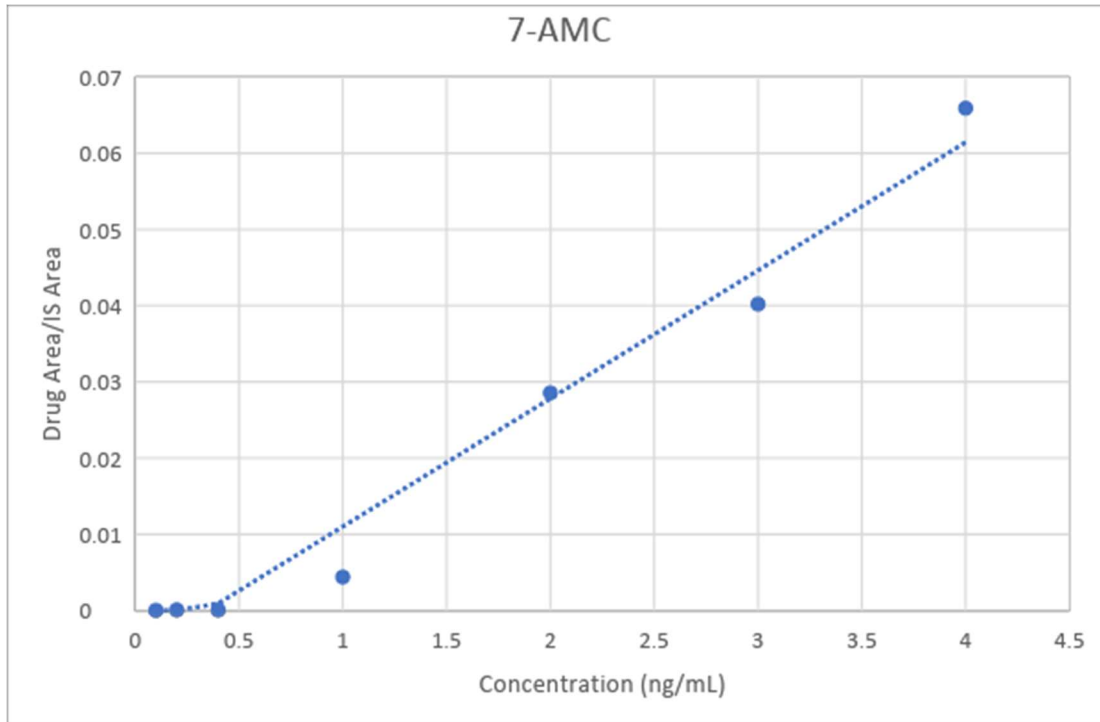| Zolpidem | | |
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.18	80.2
0.20	0.27	36.5
0.40	0.35	-12.0
1.00	0.93	-7.1
2.00	1.86	-6.8
3.00	3.07	2.2
4.00	4.04	0.9

Figure 52: 7-aminoclonazepam calibration curve run 03/16/2020. $R^2 = 0.9731$

Table 44: Bias for 7-aminoclonazepam calibration curve run 03/16/2020.

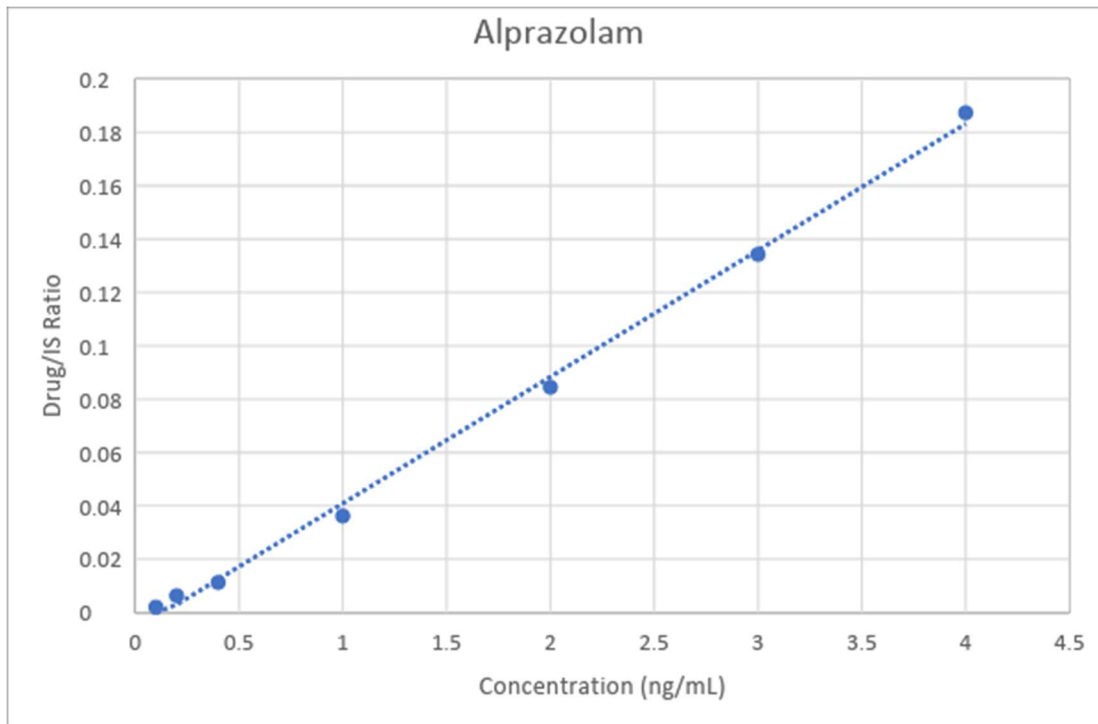| Theoretical Conc. (ng/mL)	7-aminoclonazepam Calc. Conc. (ng/mL)	Bias (%)
0.10	0.35	247.5
0.20	0.35	75.2
0.40	0.35	-12.4
1.00	0.61	-39.3
2.00	2.04	2.2
3.00	2.74	-8.7
4.00	4.27	6.7

Figure 53: Alprazolam calibration curve run 03/16/2020. R^2= 0.9973

Table 45: Bias for alprazolam calibration curve run 03/16/2020.

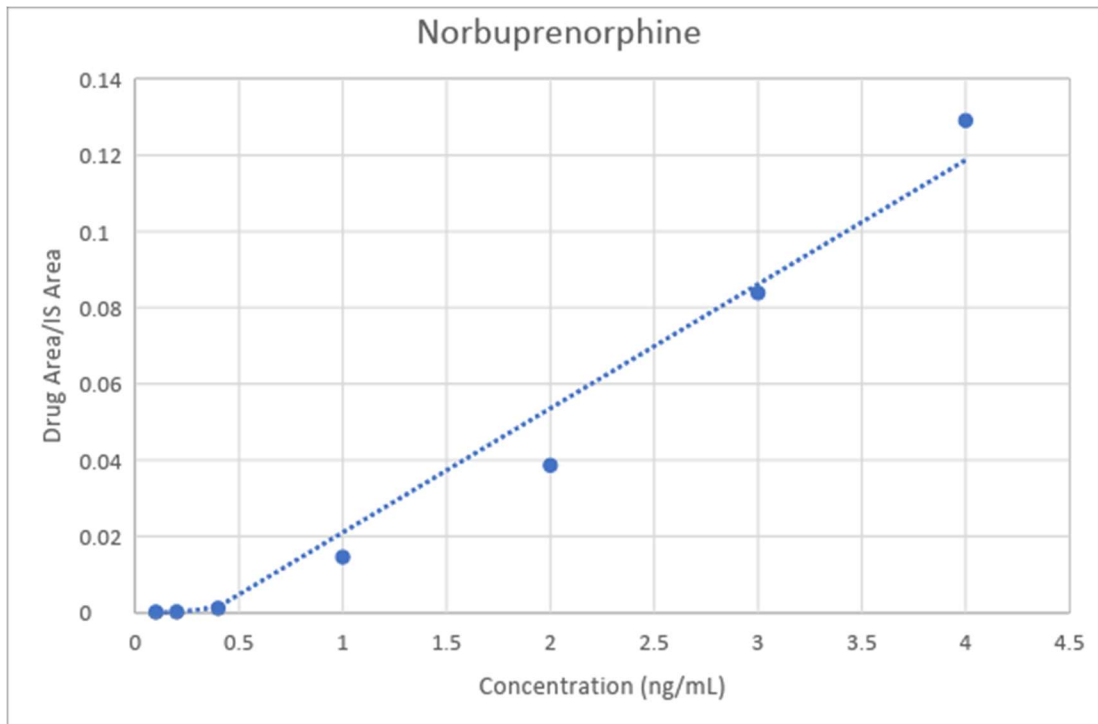|	Alprazolam	
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.18	80.1
0.20	0.27	35.1
0.40	0.37	-6.3
1.00	0.90	-9.9
2.00	1.92	-4.1
3.00	2.97	-1.0
4.00	4.09	2.2

Figure 54: Norbuprenorphine calibration curve run 03/16/2020. $R^2 = 0.9685$

Table 46: Bias for norbuprenorphine calibration curve run 03/16/2020.

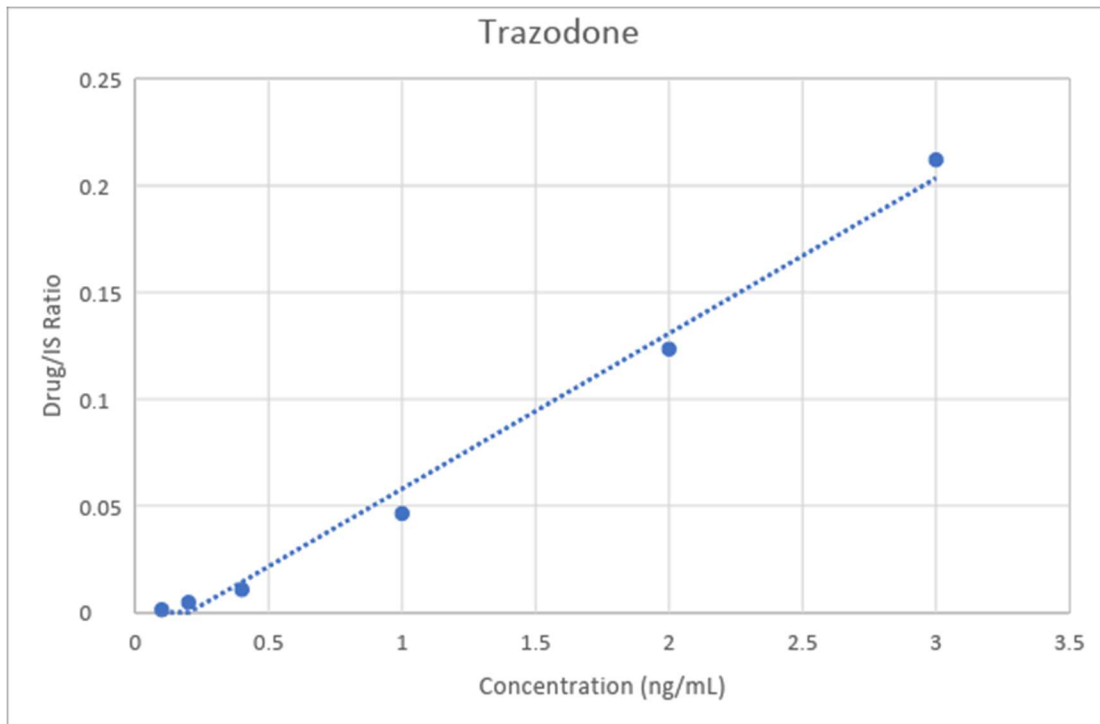| Norbuprenorphine		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.1	0.36	260.5
0.2	0.36	80.9
0.4	0.39	-2.3
1	0.8	-19.8
2	1.54	-23
3	2.93	-2.4
4	4.32	7.9

Figure 55: Trazodone calibration curve run (4.0 ng/mL point dropped due to being an outlier) 03/16/2020. R^2= 0.9895

Table 47: Bias for trazodone calibration curve run 03/16/2020.

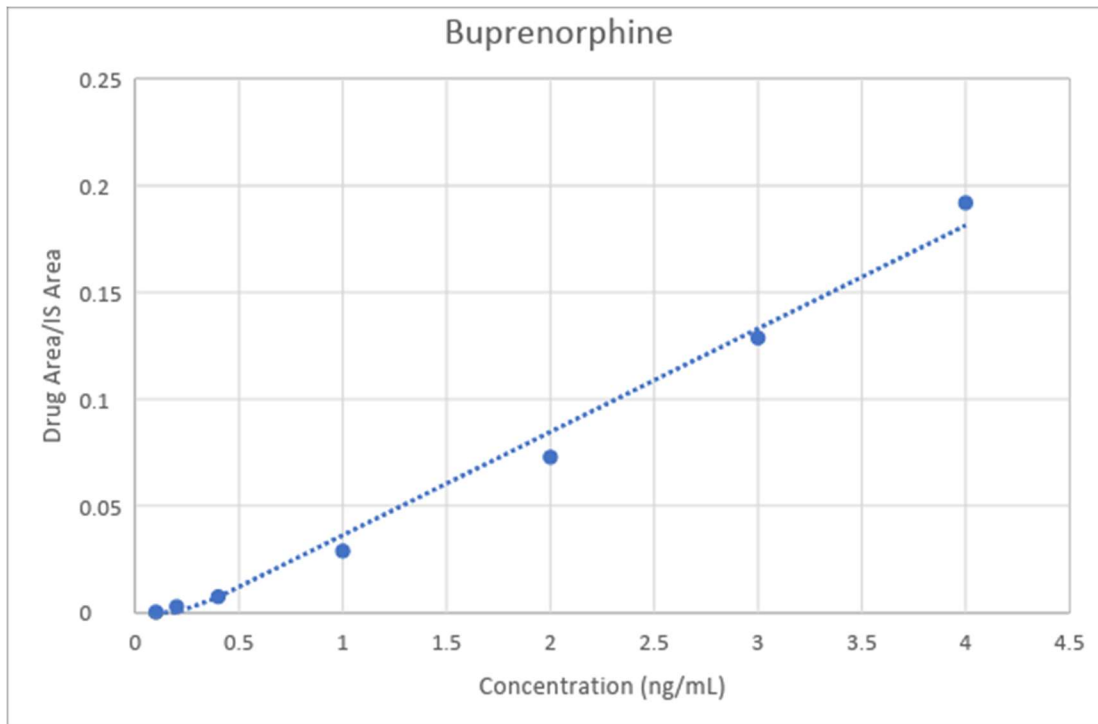| Trazodone		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.10	0.22	121.8
0.20	0.27	34.3
0.40	0.35	-12.4
1.00	0.84	-15.9
2.00	1.90	-5.0
3.00	3.12	3.9
~~4.00~~	~~5.21~~	~~30.2~~

Figure 56: Buprenorphine calibration curve run 03/16/2020. $R^2= 0.9875$

Table 48: Bias for buprenorphine calibration curve run 03/16/2020.

Buprenorphine		
Theoretical Conc. (ng/mL)	Calc. Conc. (ng/mL)	Bias (%)
0.1	0.26	157.2
0.2	0.31	54.6
0.4	0.4	1.2
1	0.85	-15.3
2	1.76	-12.2
3	2.91	-3
4	4.22	5.5

Vita

Samuel A Miller

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Master of Science in Forensic Science Drugs and Toxicology at Virginia Commonwealth University, August 2018-Present. Thesis Title: "Development of an Untargeted Gas Chromatography-Mass Spectrometry (GC/MS) Method for the Detection of Drugs in Wastewater."

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Miller, S., Rohrig, T., & Baird, T. (2018). U-47700: A Not So New Opioid. Journal of Analytical Toxicology, 42(1), E12-E14.

Miller, S., Rohrig, T., Moore, C., Stephens, K., Cooper, K., Coulter, C., Baird, T., . . . Wittman, K. (2018). Roadside drug testing: An evaluation of the Alere DDS®2 mobile test system. Drug Testing and Analysis, 10(4), 663-670.

Presentations:

Miller, S. "U-47700: A Not So New Opioid." Southwestern Association of Toxicologists Spring 2017 Meeting, Wichita, KS, April 29th, 2017.

Academic Awards:

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Southwestern Association of Toxicologists

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