DETECTION OF PRESCRIPTION AND ILLICIT DRUGS IN WASTE WATER DURING SPORTING EVENTS

By

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Bachelor of Science in Chemistry

Oklahoma State University

Stillwater, Oklahoma

2016

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2019

DETECTION OF PRESCRIPTION AND ILLICIT DRUGS IN WASTE WATER DURING SPORTING EVENTS

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ACKNOWLEDGEMENTS

Graduate school is an arduous journey that cannot be undertaken alone. This section is dedicated to the individuals who made my success possible. To begin, I am indebted to OK-LSAMP for funding my graduate studies through the bridge to doctorate program. Without their enormous support, I never would have continued my education. Next, I would like to thank my research advisor and committee chair Dr. Jarrad Wagner for providing me with the opportunity to work on this project. Without his support and guidance, this work would not have been possible. I would also like to thank Austin Ciesielski for mentoring me during my research and providing much needed advice.

Next, I would like to extend a special thank you to Dr. Ronald Thrasher for being a friend to me during graduate studies, providing much needed guidance, and always advocating for my success. Another special thank you to my peers Rachel Bonk, Kristen Lejeune, and Nichole Heneghan for being a friend to me and making this experience far more enjoyable.

In conclusion, I could not have survived this experience without the love and unconditional support of my girlfriend Yakira McCasland. My success is owed in large part to her. Lastly, I would like to dedicate this work to my late mother, Laurie Ridge, and my father Jonathan Ridge. Without my mother's foresight and strong will, I would never have made it as far as graduate school. Without my father's strength and example, I would not be the person I am today.

Name: ZACHARY RIDGE

Date of Degree: DECEMBER, 2019

Title of Study: DETECTION OF PRESCRIPTION AND ILLICIT DRUGS IN WASTE WATER DURING SPORTING EVENTS

Major Field: FORENSIC SCIENCES

Abstract: Wastewater epidemiology is a noninvasive tool that uses wastewater as a means to gather nondiscriminatory information about the exposure of a group of people to drugs, toxins, and diseases, which is accomplished by analyzing the wastewater for the analytes of interest. This study aimed to develop an analytical method for the simultaneous detection of 57 prescription and illicit drugs and their metabolites in wastewater obtained during sporting events. The epidemiological data obtained from this study can be used to inform public health and safety entities about the current use of prescription and illicit drugs in the community. Wastewater samples were obtained from a football stadium several days prior to and during a game day, extracted via solid-phase extraction, and analyzed with liquid chromatography-tandem mass spectrometry. The analytes of interest spanned several drug classes, including stimulants, opioids, benzodiazepines, and illicit drugs such as cocaine and PCP. Of the 33 samples analyzed, 28 of the 57 compounds of interest were present in at least 1 sample, with 100% of samples containing at least 1 stimulant, opioid, and illicit drug, and 24% at least 1 benzodiazepine. The findings are generally consistent with self-reporting from the community where the samples came from, and future work will include cannabinoids to detect cannabis use.

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CHAPTER I

INTRODUCTION

Pharmaceutical and illicit substance abuse is an ongoing issue within the United States. The "opioid epidemic", a colloquial term for the recent rise in opioid overdoses, is currently the focus of nationwide media attention, lawsuits against pharmaceutical companies, and proposed public policy changes. According to the Centers for Disease Control and Prevention's National Center for Health Statistics, more than 45,000 Americans died in 2017 from opioid overdoses[1]. Current methods for obtaining statistics like these are largely based on indirect estimations obtained from national surveys, police arrest and hospital records, and drug testing program reports[2,3]. One of the major sources of drug abuse statistics comes from the Substance Abuse and Mental Health Administration (SAMHSA). SAMHSA issues an annual drug abuse report containing self-reported drug use data obtained from the National Survey on Drug Use and Health and the United States Census[4].

Unfortunately, each of the aforementioned methodologies for measuring drug use is accompanied by underlying flaws, potentially leading experts to underestimate the severity of the problem. National surveys such as those used by SAMHSA, often have large gaps between successive measurements and rely on self-reporting. Personal drug use is a socially stigmatized, private activity and as such, drug abusers cannot be relied upon to accurately report their drug usage. Additionally, knowing the identity or purity of illicit substances cannot be reasonable expected of drug abusers. Hospital and police arrest records are more reliable sources of data, but only account for the small fraction of the population they encounter.

Drug testing reports, such as those issued by Quest Diagnostics, only report on the portion of individuals from which tests were obtained, are subject to manipulation by individuals being tested, and present data obtained at different intervals[5].

Thus, the complexity associated with obtaining accurate public drug abuse data has lead researchers to explore innovative techniques. For decades, environmental scientists have been aware of pharmaceutical drug contamination in our watersheds, originating from sewage networks. These trace chemicals are deposited into waste water when drugs and their metabolites are excreted from their user's bodies via urine and feces. Using well established methodologies within analytical chemistry to detect drug contamination in waste water lead researchers to develop a concept referred to as waste water-based epidemiology (WWBE).

The purpose of this work was to develop a WWBE analytical method to detect drugs of abuse and their metabolites in waste water. Waste water samples were obtained near a football stadium during a home game and 3 days prior to the game. The hypothesis being tested is that drugs detected in waste water will be elevated when stadiums are in use for a special event with a larger population present. The data obtained from analyzing these samples was used to determine whether drug concentrations in waste water are elevated during football games when compared to times when the stadium is not in use. Additionally, this study serves a proof of concept for the single extraction and analysis of waste water for various drugs abuse spanning multiple drug classes.

CHAPTER II

REVIEW OF LITERATURE

2.1 History

While the presence of pharmaceutical drugs in waste water treatment plants (WWTP) and surface waters was established in the 1990s, the presence of illicit drugs and their metabolites was not seriously considered until 2001 when Christian G. Daughton first proposed monitoring waste water as a method for obtaining insight on the public's use of controlled substances[2,6–8]. Daughton argued that monitoring WWTP offered an "unobtrusive, non-invasive" approach to surveying a communities controlled substance intake and eventually provide social scientists with real-time data[2]. Zuccato et al., the first researchers who attempted Daughton's waste water analysis approach, analyzed composite waste water samples collected from multiple Italian WWTP for cocaine and its metabolite, benzoylecgonine[9]. The results of this initial study were promising, with estimates revealing a far larger incidence of cocaine use than traditional methods[9]. After receiving worldwide attention for the initial study, Zuccato et al. published a follow-up study in 2006 looking for amphetamines, cannabinoids, and opioids in waste water collected from Italian and Swiss WWTP, with similar results[10].

These initial successes spawned thousands of additional studies across the globe. In April 2007, the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) held the first major conference among experts in drug epidemiology and WWBE to encourage collaboration[11].

Soon after in 2010, an international European research group, Sewage Analysis Core Group Europe (SCORE), was formed between institutions in Belgium, Switzerland, Spain, Italy, the Netherlands, Norway, and the United Kingdom[12]. Since its inception, SCORE has organized several large scale case studies in an attempt to develop standardized techniques, making it easier to compare relevant data and identify common issues[3]. An important aspect of SCORE is their publication of ethical guidelines related to using WWBE. The most recently published of these studies targeted 7 illicit drug residues and spanned 6 years, 4 continents, and 25 countries[12].

Following the formation of SCORE and subsequent groundbreaking studies, the EMCDDA held a second interdisciplinary conference in October 2015 to highlight the exponential growth and progress being made within WWBE[3]. Unlike the 2007 conference, which focused mainly on the development of the still novel WWBE approach, the 2015 conference's mainly focused on the various issues plaguing WWBE[3,11]. Sampling and analysis technique, fluctuations in water flow, drug stability in waste water, back-calculation of drug use, and population size estimates were among the main uncertainties outlined in the 2015 conference[3]. As a result, many researchers are now focused on identifying and addressing these uncertainties.

2.2 Waste Water Analysis During Special Events

The most common application of WWBE has been to estimate local illicit drug use within the larger community[3]. Recently, the focus of many researchers has narrowed to smaller, controlled populations. Traditional epidemiological techniques have identified trends of increased substance abuse during weekends, seasonal changes, and special events. Understanding these temporary fluctuations in analyte concentration is a crucial factor in translating epidemiological data obtainable from large composite samples[13]. Additionally, using WWBE to further explore the impacts of these events can provide information that self-reporting cannot reliably predict,

such as the presence of emerging illicit psychoactive substances like mephedrone and benzylpiperazine[14].

One of the first major publications to explore these temporal variations, conducted over several years in Spain by Huerta-Fontela et al., revealed elevated concentrations of cocaine and amphetamine-type stimulants during weekends and the summer and winter seasons[15]. Later Metcalfe et al. conducted the first WWBE study in Canada, finding further evidence of a trend of increased cocaine consumption on weekends[16]. Lai et al. provided a more detailed profile of illicit substance variations in Austrian waste waters during Christmas and New Year's, finding elevated cocaine and MDMA concentrations but baseline cannabis and methamphetamine excretion[17].

The first example of WWBE being applied at special events was in 2009 when Bijlsma et al. analyzed waste water effluent collected from an "important rock event", finding elevated levels of Methylenedioxy-methamphetamine (MDMA) and the cocaine metabolite benzoylecgonine[18]. A similar trend of drug abuse at festivals was identified earlier in Australia by a cross-sectional survey correlating attendance of a music festival in Melbourne and higher illicit drug use than the general population[19]. Lai et al. later confirmed this trend in Australia by analyzing waste water obtained at an annual Australian music festival, finding a steady increase in cannabis, MDMA, methamphetamine, and cocaine use over several days in two separate years[14].

Sports tournaments such as the annual American football championship, the Super Bowl, have also been shown to influence illicit substance consumption[13]. In 2010 Gerrity et al. compared WWTP samples collected near a major United States city during Super Bowl weekend and a normal weekend[13]. They found that cocaine use increased during the Super Bowl while methamphetamine use slightly decreased[13]. Football stadiums provide the unique opportunity to observe the influence of tourism from the opposing team, previously identified weekend

increases, and the impact of special event attendance. Furthermore, there are less unknown variables involved since population can be determined by ticket sales and increases in flow rates are more predictable (e.g. half-time). Gul et al. were the first to analyze effluent from a football stadium located at the University of Mississippi in a groundbreaking four part study[20]. Each part of the study focused on developing a method to analyze the same wastewater samples for the different classes of commonly abused substances: stimulants, opiates, benzodiazepines, and miscellaneous drugs[20–23]. In each study, increases in drug concentrations were observed during games and variations occurred depending on which away team was playing[20–23].

2.3 Laboratory Techniques

The majority of WWBE studies use solid phase extraction (SPE) to isolate and concentrate compounds prior to analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Another common method of analysis is gas chromatography-mass spectrometry (GC-MS). However, GC-MS is quickly being replaced as the gold standard for toxicological analysis due to recent advances in LC-MS/MS technology.

2.3.1 Solid Phase Extraction

Waste water by nature contains several contaminants that can interfere with the detection of any drugs and metabolites that might be present. Additionally, the volume of water present in sewage systems dilutes the analytes of interest significantly, making them difficult to detect. SPE is a multi-step process by which sample is passed through a column containing a sorbent bed of micro-particles, 20-40 microns in size[24]. These micro-particles are typically composed of hydrocarbon chains, phenyl rings, and positively and negatively charged sites bound to silica[24]. As the aqueous sample passes through the sorbent bed, polar organic compounds suspended in solution such as drugs and metabolites bind to micro-particles, allowing water and undesirable compounds to flow through as waste[24]. The sorbent can then be washed to remove any unwanted

materials caught in the sorbent and dried to remove excess water. The sorbent-bound analytes can then be eluted using an appropriate organic solvent[24]. Lastly, to further concentrate the elution, organic solvent can be evaporated off by applying pressure via an inert gas such as argon or nitrogen, and the eluent can be resuspended in a smaller volume of solvent that is appropriate to the method of analysis.

2.3.2 Liquid Chromatography

The principles of liquid chromatography (LC) are similar to SPE in that a column containing micro-particles is used to separate chemical compounds. However, in the case of LC, micro-particles referred to as the stationary phase are packed throughout a temperature controlled column in which a pressurized liquid mobile phase flows through[24,25]. Modern LC columns use far smaller particle sizes than SPE, less than 10 microns, to achieved greater analyte separation[25]. The use of such small particles generates immense pressure to achieve desirable mobile phase flow rates, thus most modern LC is high pressure liquid chromatography (HPLC)[25].

Analyte separation is achieved based on a chemical's polarity and subsequent affinity to the stationary phase. For sample matrices in which analytes have very different affinities, isocratic elution can be used to achieve separation[25]. However, for screens containing analytes with similar polarities, a gradient elution is necessary for adequate separation. Gradient elutions are the most widely used HPLC method for WWBE, as researchers are often interested in a wide range of compounds.

In a gradient elution the mobile phase is composed of a mixture of solvents that's composition changes throughout the run, such as methanol and water[25]. During sample injection onto the column, the mobile phase is kept at a low elution strength, allowing analytes to become trapped by the stationary phase[25]. Once loaded, the gradient will change to begin eluting compounds with the least affinity toward the stationary phase first, followed by more strongly

retained analytes[25]. The time it takes for an analyte to move through the column is referred to as the compound's retention time [25]. Retention times are useful in determining if a suspected analyte is present but cannot be used for absolute identification, as multiple compounds can have the same or very similar retention times[25].

2.3.3 Tandem Mass Spectrometry

Mass spectrometry (MS) is an analysis technique used to identify chemicals according to their mass-to-charge ratios (m/z)[26]. This is achieved by ionizing compounds in their gaseous phase, typically by either adding ($M+H^+$) or removing a proton ($M-H^+$)[24,26]. Molecules are ionized so they can be easily manipulated by electrostatic and magnetic fields, allowing them to be isolated and measured[24]. Ions then enter the mass analyzer, a vacuum chamber containing four parallel metal rods in which radio frequency (RF) and direct current (DC) voltages are applied, called a quadrupole[24,26]. These RF and DC potentials are used to filter ions by their m/z, allowing only ions within a specific m/z range to reach the detector and produce a signal[24,26].

Tandem mass spectrometry (MS/MS) is a form of multistage MS analysis that allows for better analyte identification[24,26]. The most common form of MS/MS used for WWBE is multiple reaction monitoring (MRM). In MRM, three mass analyzers are aligned in sequence with the first and last acting as mass filters and the central analyzer functioning as a collision cell[24]. The first quadrupole, Q1, functions just like a traditional MS, filtering ionized compounds by their m/z. These ions, referred to as precursor ions, then pass into the collision cell, Q2. The Q2 consists of a quadrupole, hexapole, octapole, or other design filled with an inert collision gas, typically argon or nitrogen[24]. Precursor ions collide with collision gas molecules and fragment into product ions with different m/z. The third analyzer, Q3, filters the product ions within a specified m/z range. MS/MS allows for more accurate analyte identification than traditional MS because precursor ions of similar m/z are not likely to fragment in exactly the same way, thus producing easily

identified product ions. Additionally, even if similar product ions are produced, the ratio in which they are produced is likely to be different for different precursor ions. For example, morphine and hydromorphone both have a m/z of 286 but morphine's product ions are 165 and 152 whereas hydromorphone's product ions are 184 and 157. Isolating these ions would be difficult on a traditional single quadrupole MS, but is far easier after fragmentation on MS/MS.

2.4 Compounds of Interest

The LC-MS/MS method developed for this study screens for the following 56 drugs and metabolites: 6-monoacetylmorphine (6-MAM), 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxytriazolam, alprazolam, amphetamine, benzoylecgonine, buprenorphine, carfentanil, carisoprodol, cocaine, codeine, cyclobenzaprine, desalkylflurazepam, diazepam, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), fentanyl, flunitrazepam, flurazepam, hydrocodone, hydromorphone, ketamine, lorazepam, MDMA, meperidine, meprobamate, methadone, methamphetamine, methylphenidate, midazolam, morphine, naloxone, naltrexone, norbuprenorphine, norcarfentanil, nordiazepam, norfentanyl, norhydrocodone, normeperidine, noroxycodone, O-desmethyltramadol, oxazepam, oxycodone, oxymorphone, phencyclidine (PCP), phentermine, propoxyphene, pseudoephedrine, sufentanil, tapentadol, temazepam, tramadol, trazodone, triazolam, and zolpidem. Δ^9-Tetrahydrocannabinol (THC), and 11-nor-9-carboxy-Δ^9-THC (THCA) were originally included in the study but were not successfully extracted from wastewater and thus excluded in the final results.

2.4.1 Opioids

Opioid is an overarching term referring to naturally occurring alkaloid analgesics derived from the opium poppy, semisynthetic alkaloids derived from naturally occurring opiates, and synthetic compounds that mimic the pharmacological effects of opiates[24]. The pharmacological effects of opioids are generally shared across all subclasses and impact the central nervous system

(CNS)[24]. While most opioids are used clinically for their analgesic effects, they have a high abuse potential due to their euphoric and sedative psychoactive effects[24]. Overdoses ending in death are often the result of respiratory failure[24].

Two of the three naturally occurring opiates, morphine and codeine, are included in this studies analysis panel in addition to the semisynthetic opioids hydrocodone, norhydrocodone, hydromorphone, oxycodone, noroxycodone, and oxymorphone[27]. 6-MAM, the metabolite of the semisynthetic opioid heroin has been reserved for the illicit section, 2.4.4, as the United States Drug Enforcement Agency (DEA) classifies it as a schedule I substance, meaning it has no accepted medical use[28]. The following synthetic opioids and their metabolites are also included in the panel: buprenorphine, norbuprenorphine, carfentanil, norcarfentanil, fentanyl, norfentanyl, sufentanil, methadone, EDDP, meperidine, normeperidine, naloxone, naltrexone, propoxyphene, tapentadol, tramadol, and O-desmethyltramadol.

2.4.2 Benzodiazepines

Benzodiazepines are one of the most commonly prescribed drug classes in the United States and act as a CNS depressant[24]. First synthesized from an accidental reaction between quinazoline N-oxide and methylamine in the 1930's, benzodiazepines were approved for clinical use by the FDA in 1960 and have largely replaced another CNS-depressant drug class, barbiturates, due to a reduced risk of adverse side effects and overdose[24]. One of the most common therapeutic uses of these drugs is as an anxiolytic, or to treat anxiety disorders[24]. The benzodiazepines included in this study are alprazolam, α-hydroxyalprazolam, 7-aminoclonazepam, diazepam, nordiazepam, oxazepam, temazepam, flurazepam, lorazepam, midazolam, triazolam, and α-hydroxytriazolam. The benzodiazepine flunitrazepam and its metabolite are not included in this section since they are classified as schedule IV substances in the United Stated and will instead be discussed under section 2.4.4[28].

2.4.3 CNS Stimulants

CNS stimulants, as the name would imply are compounds that bind to endogenous neurotransmitters to stimulate the central nervous system. CNS stimulants act through raising dopamine and norepinephrine levels within the body which elevates blood pressure, stimulates respiratory action, and at therapeutic doses raises the heart rate[24,29]. Many CNS stimulants have been identified as dangerous due to their potential for abuse and addiction due to their euphoric effects[24]. The most common cause of death by CNS stimulants are cardiac complications brought on by consumption of high doses[24].

The CNS stimulants of interest to this study are all amphetamines with the exception of methylphenidate, a drug commonly prescribed for attention deficit disorder under the brand name Ritalin. Cocaine, MDMA, and methamphetamine are also CNS stimulants of interest to this study but will be discussed in section 2.4.5 since MDMA is a schedule I substance and cocaine and methamphetamine are schedule II substances[24,28]. Amphetamine, phentermine, and pseudoephedrine (a decongestant and methamphetamine precursor) are the stimulants of interest to this study.

2.4.4 Illicit Drugs

For the purposes of this study, illicit drugs are compounds listed as schedule I controlled substances by the DEA or schedule II substances rarely used therapeutically such as cocaine, ketamine, and methamphetamine. The illicit substances of interest to this study are cocaine, flunitrazepam, ketamine, MDMA, methamphetamine, and PCP. The metabolite of the schedule I opioid heroin, 6-MAM, was included in this study while the parent compound was left out since it is typically quickly and entirely metabolized. The metabolites of cocaine (benzoylecgonine) and flunitrazepam (7-aminoflunitrazepam) are also included in this study.

2.4.5 Miscellaneous Drugs

The miscellaneous drugs of interest to this study are compounds which do not fall under any of the other drug classes listed above. They include the muscle relaxants carisoprodol and cyclobenzaprine, the tricyclic antidepressant trazodone, and the hypnotic zolpidem. The metabolite of carisoprodol that is also sold as a drug, meprobamate, is also included in this study. The effects of each of these drugs is most similar to that of a benzodiazepine.

2.5 Conclusion

The focus of this research is to develop a LC-MS/MS method for analyzing wastewater samples taken during football games. Specifically, the analysis method in question will screen for a panel of both prescription and illicit drugs and their metabolites from a wide range of drug classes. Previous studies have mainly focused on only one drug class or category at a time, developing relatively small analysis panels. Those studies which chose to screen for a large panel of substances did not apply them to special events. This study intends to provide a proof of concept for both the ability to detect numerous compounds in a single analysis and apply those findings to establishing drug trends within a population attending special events.

CHAPTER III

METHODOLOGY

3.1 Sampling Strategy

Thirty-three (33) wastewater samples, listed in Table 1, were manually collected by the University of Florida (UF) collaborators during the football game between the Florida Gators and Kentucky Wildcats on Saturday, September 8, 2018 (game day population of 80,651). Samples were collected near the Ben Hill Griffith Stadium at three discrete locations to examine the variability in drug concentrations according to sub-populations (home, away, student, etc.) that attended the game. Samples per site were collected every half hour beginning approximately an hour before kickoff to 30 minutes after kickoff. Each sample was collected and placed into three different tubes: one 50 mL tube, and two 15 mL tubes. The continuous sampling strategy was designed to ensure comprehensive collection throughout the entire game, as well as enable the tracking of concentrations of drugs throughout the game. Four (4) additional samples were collected from the same locations and a wastewater station on Wednesday, September 5, 2018 to serve as background specimens. Once collected, samples were placed into a freezer for storage and mailed to the Oklahoma State University Forensic Toxicology and Trace Laboratory (OSU-FTTL) for analysis.

Table 1. A complete list of the wastewater samples obtained by the University of Florida. Each sample was assigned a number for ease of reference. The date, time, and location of collection are listed in each column. The time of collection for the background samples was not provided.

Sample #	Date (Month/Day/Year)	Time	Location
Background 1	09/05/2018	Unknown	University Ave
Background 2	09/05/2018	Unknown	Wastewater Station
Background 3	09/05/2018	Unknown	Pump Station
Background 4	09/05/2018	Unknown	Gale Lemerand Ave
1	09/08/2018	6:30	University Ave
2	09/08/2018	6:30	Pump Station
3	09/08/2018	6:30	Gale Lemerand Ave
4	09/08/2018	7:00	University Ave
5	09/08/2018	7:00	Pump Station
6	09/08/2018	7:00	Gale Lemerand Ave
7	09/08/2018	7:30	University Ave
8	09/08/2018	7:30	Pump Station
9	09/08/2018	7:30	Gale Lemerand Ave
10	09/08/2018	8:00	University Ave
11	09/08/2018	8:00	Pump Station
12	09/08/2018	8:00	Gale Lemerand Ave
13	09/08/2018	8:30	University Ave
14	09/08/2018	8:30	Pump Station
15	09/08/2018	8:30	Gale Lemerand Ave
16	09/08/2018	9:00	University Ave
17	09/08/2018	9:00	Pump Station
18	09/08/2018	9:00	Gale Lemerand Ave
19	09/08/2018	9:30	University Ave
20	09/08/2018	9:30	Pump Station
21	09/08/2018	9:30	Gale Lemerand Ave
22	09/08/2018	10:00	University Ave
23	09/08/2018	10:00	Pump Station
24	09/08/2018	10:00	Gale Lemerand Ave
25	09/08/2018	10:30	University Ave
26	09/08/2018	10:30	Pump Station
27	09/08/2018	10:30	Gale Lemerand Ave
28	09/08/2018	11:00	University Ave
29	09/08/2018	11:00	Pump Station
30	09/08/2018	11:00	Gale Lemerand Ave
31	09/08/2018	11:30	University Ave
32	09/08/2018	11:30	Pump Station
33	09/08/2018	11:30	Gale Lemerand Ave

3.2 Chemicals and reagents

All materials were purchased from commercial suppliers except Nanopure water, which was obtained using a Barnstead Nanopure Diamond laboratory water system (Thermo Scientific, Waltham, MA). ACS-grade 37% hydrochloric acid was purchased from BDH (BDH Scientific, Radnor, PA). LC-MS grade methanol and ACS-grade ammonium hydroxide were purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). ACS-grade isopropyl alcohol was purchased from EM Science (EM Science, Gibbstown, NJ). HPLC grade dichloromethane was purchased from VWR (VWR Analytical, Sugar Land, TX). HPLC grade 98% formic acid was purchased from EMD (EMD Millipore Corporation, Billerica, MA). Crystalline ammonium formate (99%) was purchased from Alfa Aesar (Alfa Aesar, Ward Hill, MA).

Drug standards at a concentration of 1 mg/mL in methanol were mostly purchased from Lipomed (Lipomed Inc, Cambridge, MA). EDDP, fentanyl, and norbuprenorphine drug stocks were also purchased from Lipomed at a concentration of 100 µg/mL. Methylphenidate and nordiazepam drug standards were purchased from Cerilliant (Cerilliant Corp, Round Rock, TX) at 1 mg/mL and sufentanil was purchased from Cerilliant at 100 µg/mL in methanol. Carfentanil and norcarfentanil were purchased from Cayman (Cayman Chemical, Ann Arbor, MI) at a concentration of 100 µg/mL in methanol. 7-Aminoclonazepam-D4, buorenorphine-D4, carisoprodol-D7, cocaine-D3, morphine-D6, nordiazepam-D5, and normeperidine-D4 deuterated internal standards were purchased from Cerilliant at a concentration of 1 mg/mL in methanol. All other deuterated internal standards were purchased from Cerilliant at a concentration of 100 µg/mL in methanol.

3.3 Solution Preparation

Separate calibration stock solution and quality control (QC) stock solution containing all of the analytes of interest at a concentration of 2500 ng/mL were prepared in methanol using the drug standards described in section 3.3. The details of this preparation are seen in Table 2. An additional internal standard stock solution containing 2500 ng/mL of each deuterated compound was prepared as seen in Table 3. All 3 stock solutions were created by spiking methanol with an aliquot of each certified drug standard.

Table 2. Preparation for 2 mL of calibration and QC stock solution from certified drug standards. Spike volumes were calculated according to the µg/mL drug standard concentration in order to reach a final concentration of 2500 ng/mL.

Analyte Name	Standard Concentration (µg/mL)	Spike Volume (µL)	Final Concentration (ng/mL)
6-MAM	1000	5	2500
7-Aminoclonazepam	1000	5	2500
7-Aminoflunitrazepam	1000	5	2500
A-Hydroxyalprazolam	1000	5	2500
A-Hydroxytriazolam	1000	5	2500
Alprazolam	1000	5	2500
Amphetamine	1000	5	2500
Benzoylecgonine	1000	5	2500
Buprenorphine	1000	5	2500
Carfentanil	100	50	2500
Carisoprodol	1000	5	2500
Cocaine	1000	5	2500
Codeine	1000	5	2500
Cyclobenzaprine	1000	5	2500
Desalkylflurazepam	1000	5	2500
Diazepam	1000	5	2500
EDDP	100	50	2500
Fentanyl	100	50	2500
Flunitrazepam	1000	5	2500
Flurazepam	1000	5	2500
Hydrocodone	1000	5	2500
Hydromorphone	1000	5	2500
Ketamine	1000	5	2500
Lorazepam	1000	5	2500

Analyte Name	Standard Concentration (µg/mL)	Spike Volume (µL)	Final Concentration (ng/mL)
MDMA	1000	5	2500
Meperidine	1000	5	2500
Meprobamate	1000	5	2500
Methadone	1000	5	2500
Methamphetamine	1000	5	2500
Methylphenidate	1000	5	2500
Midazolam	1000	5	2500
Morphine	1000	5	2500
Naloxone	1000	5	2500
Naltrexone	1000	5	2500
Norbuprenorphine	100	50	2500
Norcarfentanil	100	50	2500
Nordiazepam	1000	5	2500
Norfentanyl	1000	5	2500
Norhydrocodone	1000	5	2500
Normeperidine	100	50	2500
Noroxycodone	1000	5	2500
O-Desmethyltramadol	1000	5	2500
Oxazepam	1000	5	2500
Oxycodone	1000	5	2500
Oxymorphone	1000	5	2500
PCP	1000	5	2500
Phentermine	1000	5	2500
Propoxyphene	1000	5	2500
R, R Pseudoephedrine	1000	5	2500
Sufentanil	100	50	2500
Tapentadol	1000	5	2500
Temazepam	1000	5	2500
THCA	1000	5	2500
Tramadol	1000	5	2500
Trazodone	1000	5	2500
Triazolam	1000	5	2500
Zolpidem	1000	5	2500
THC	1000	5	2500

Total Spike Volume (µL) = 605
Methanol Volume (µL) = 1395
Total Solution Volume (µL) = 2000

Table 3. Preparation for 2 mL of deuterated internal standard stock solution from certified deuterated drug standards. Spike volumes were calculated according to the µg/mL internal standard concentration in order to reach a final concentration of 2500 ng/mL.

Analyte Name	Internal Standard Concentration (µg/mL)	Spike Volume (µL)	Final Concentration (ng/mL)
7-Aminoclonazepam-D4	1000	5	2500
7-Aminoflunitrazepam-D7	100	50	2500
A-Hydroxytriazolam-D4	100	50	2500
Amphetamine-D5	1000	5	2500
Benzoylecgonine-D3	100	50	2500
Buorenorphine-D4	1000	5	2500
Carisoprodol-D7	1000	5	2500
Cocaine-D3	100	50	2500
Codeine-D6	1000	5	2500
Cyclobenzaprine-D3	100	50	2500
Diazepam-D5	100	50	2500
Fentanyl-D5	100	50	2500
Flunitrazepam-D7	100	50	2500
Hydromorphone-D3	1000	5	2500
Meperidine-D4	1000	5	2500
Methadone-D3	1000	5	2500
Methamphetamine-D5	1000	5	2500
Methylphenidate-D9	100	50	2500
Morphine-D6	1000	5	2500
Nordiazepam-D5	1000	5	2500
Normeperidine-D4	100	50	2500
Oxycodone-D6	1000	5	2500
PCP-D5	1000	5	2500
Propoxyphene-D5	100	50	2500
Pseudoephedrine-D3	1000	5	2500
THCA-D3	1000	5	2500

Total Spike Volume (µL) = 625
Methanol Volume (µL) = 1375
Total Solution Volume (µL) = 2000

From the 2500 ng/mL calibration stock solution, eight (8) calibrators of the concentrations outlined in Table 4 were prepared in nanopure water via serial dilution using the

steps outlined in Table 5. 2 QCs were also prepared in a similar fashion using the 2500 ng/mL QC stock solution, outlined in Table 6.

Table 4. Name and concentration of the 8 calibrators prepared via serial dilution from the 2500 ng/mL calibration stock.

Pre-SPE Concentration (ng/mL)	Calibrator Name
20	400c
10	200c
5	100c
2.5	50c
1.25	25c
0.75	15c
0.50	10c
0.05	1c

Table 5. Serial dilution preparation of 8 calibration solutions in water from a 2500 ng/mL calibration stock.

Cal. Name	Stock (µL)	400c (µL)	200c (µL)	100c (µL)	50c (µL)	25c (µL)	15c (µL)	10c (µL)	Water (uL)	Total (µL)	Final (uL)
400c	104								12896	13000	6470
200c		6530							6530	13060	6515
100c			6545						6545	13090	6505
50c				6585					6585	13170	6500
25c					6670				6670	13340	6560
15c						6780			4520	11300	6500
10c							4800		2400	7200	6550
1c								650	5850	6500	6500

Table 6. Serial dilution preparation of 2 QCs in water from a 2500 ng/mL QC stock.

Concentration (ng/mL)	Stock (µL)	5 ng/mL (µL)	Water (uL)	Total Volume (µL)	Remaining (uL)
5	15		7485	7500	6525
0.75		975	5525	6500	6500

3.4 Solid phase extraction

SPE was performed on the wastewater samples, the 8 calibrators, and a blank consisting only of nanopure water using a SPEware Cerex 48 positive pressure manifold and sample concentrator, seen in Figure 1 (Tecan SP, Inc., Baldwin Park, CA). Tecan Cerex Trace-B, 6mL columns, 50mg cartridges (Tecan SP, Inc., Baldwin Park, CA) were conditioned with 2 mL of methanol, followed by 2 mL of Nanopure water, and then 2 mL of pH 5 Nanopure water. Prior to loading each sample onto the SPE cartridge, 10 µL of internal standard mix and 25 µL of 100 mM HCl was added to 6 mL of sample and the mixture was vortexed. Following sample addition, the SPE cartridges were washed twice with 2 mL of pH 5 Nanopure water. The cartridges were then dried under 70 psi nitrogen for 20 min. Following the drying step, 2 mL of an elution solution containing 80:18:2 (dichloromethane: isopropyl alcohol: ammonium hydroxide) was added to each cartridge twice and collected in a test tube. The elution mixtures were dried to complete dryness under a stream of nitrogen at 40 °C and reconstituted in 100 µL of 98% mobile phase A and 2% mobile phase B, the starting conditions of the LC gradient, before being transferred to LC injection vials for instrumental analysis. An outline of this procedure is given in Table 7.

Figure 1. A picture of the SPEware Cerex 48 positive pressure manifold and sample concentrator used to perform SPE on the wastewater samples. On the left is the positive pressure manifold used to applying nitrogen gas for loading samples and drying the SPE cartridge sorbent. On the right is the sample concentrator used to accelerate the evaporation of the elution mixture.

Table 7. Outline of the solid phase extraction procedure.

SPE Step	Parameter
Sample Preparation	6 mL wastewater 10 μL 2500 ng/mL internal standard mix 25 μL 100mM HCl
Condition	2 mL LC-MS grade methanol 2 mL Nanopure water 2 mL pH 5 Nanopure water
Sample Addition	6.035 mL sample
Rinse	2 x 2 mL pH 5 Nanopure water
Cartridge Dry Down	20 min at ~70 psi
Elution	2 x 2 mL 80:18:2 dichloromethane:isopropanol:ammonium hydroxide
Elution Dry Down	Under nitrogen at 40ºC
Reconstitution	100 μL 98:2 mobile phase A:mobile phase B

3.5 LC-MS/MS Analysis

The 4 background samples were extracted and analyzed once due to the sample volume available, while the other 33 samples were extracted on two separate occasions. The first extraction was analyzed on a Waters Acquity Classic UPLC-MS/MS system. During this initial analysis a number of QCs and calibrators were observed outside of acceptable parameters, and therefore the analysis was repeated on the Waters system, with similar results. These samples were then moved to a Shimadzu UFLC-MS/MS system in the OSU-FTTL for further analysis in order to rule out issues specifically related to the Waters method.

3.5.1 Waters Acquity Classic UPLC-MS/MS

Initially a method for instrumental analysis on a Waters Acquity Classic UPLC-MS/MS system, seen in Figure 2, was developed for this project. Separation was achieved using a Waters Cortecs C18 HPLC column (2.1 x 100 mm; 2.7 um; 90 Å) with an Acquity UPLC BEH C18 1.7 μm guard cartridge (2.1 x 5mm) attached (Waters Corporation, Milford, MA). Mobile phase A consisted of 10mM ammonium formate and 0.1% formic acid in water and mobile phase B

consisted of 10 mM ammonium formate and 0.1% formic acid in methanol. The LC pumps were held at a flow rate of 0.400 mL/min and column temperature was maintained at 65 °C. Sample injections were set at 10 µL. LC-MS/MS methods were developed and chromatograms were observed using Mass Lynx (Waters Corporation, Milford, MA). Analyte quantification was done using Target Lynx (Waters Corporation, Milford, MA).

Figure 2. A picture of the Waters Acquity Classic UPLC-MS/MS system used to analyze the wastewater samples in this study. On the right are the UPLC pumps, autosampler, and column oven. On the left is the tandem quadrupole detector (TQD).

The gradient program used for chromatographic separation of the compounds of interest, visualized graphically in Figure 3, began at 2% mobile phase B and was maintained at 2% for 1.25 min before increasing to 20% in 3.75 min. The mobile phase B concentration was then increased to 50% in 4 min, followed by a further increase to 90% mobile phase B in 1 min. The

gradient was then held at 90% for 0.5 minutes before being returned to 10% mobile phase B in 1.5 min. The gradient was further decreased to 2% mobile phase B in 0.01 min and held for the last minute, resulting in a total run time of 13 minutes.

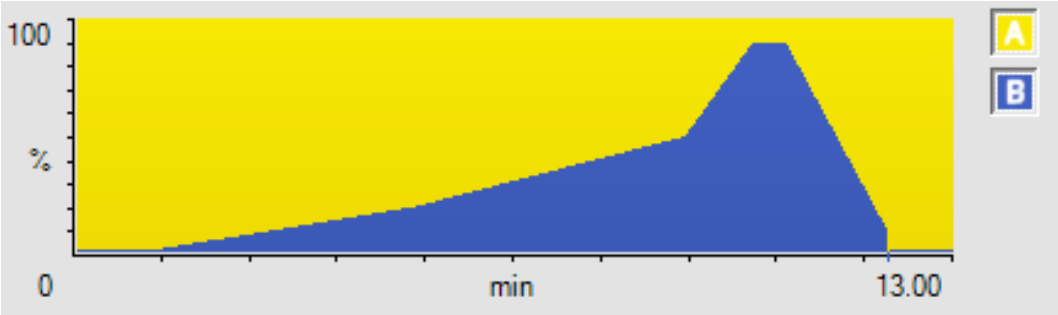

Figure 3. A plot of the chromatographic gradient used to separate the compounds of interest presented as percentage of each mobile phase over time. The yellow color represents mobile phase A while the blue represents mobile phase B.

MRM ion transitions and parameters were optimized using Mass Lynx Intellistart. These parameters are summarized in Table 8 for the compounds of interest and Table 9 for the deuterated internal standards. Compound identity was confirmed by use of an MRM ratio, which compared the MRM transition with a smaller peak area to the MRM transition with a larger peak area. An acceptable range for the MRM ratio was calculated by averaging the MRM ratios of each calibrator for a given compound. The MRM ratios had to be within 20% of this averaged MRM ratio. Identification was further established by relative retention times. Relative retention times were defined as the retention time of a compound of interest divided by the retention time of said compound's internal standard. As with the MRM ratios, an acceptable range was determined by averaging the relative retention times of the calibrators for a given compound; all peaks were required to be within 2.5% of this averaged relative retention time.

Table 8. Retention times, MRM transitions, and optimized mass spectrometer parameters used to analyze for compounds of interest on the Waters Acquity Classic UPLC-MS/MS. For each compound, the quantitative MRM transition is on the top row and the qualitative MRM transition is on the second row.

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	Cone (V)
6-MAM	4.39	328.16	165.01	36	17
		328.16	57.97	30	17
7-Aminoclonazepam	5.92	286.04	120.97	28	50
		286.04	222.03	24	50
7-Aminoflunitrazepam	6.90	284.08	135.11	30	48
		284.08	226.39	34	48
Alpha-Hydroxyalprazolam	9.62	325.03	297.19	24	51
		325.03	216.05	40	51
Alpha-Hydroxytriazolam	9.54	359.07	176.09	26	54
		359.07	331.04	28	54
Alprazolam	9.95	308.97	281.02	26	31
		308.97	204.93	40	31
Amphetamine	3.81	135.95	91.10	16	22
		135.95	119.13	10	22
Benzoylecgonine	5.39	290.23	168.10	18	39
		290.23	105.02	28	39
Buprenorphine	9.22	468.29	54.98	52	44
		468.29	83.79	44	44
Carfentanil	8.57	395.40	113.09	32	32
		395.40	335.25	18	32
Carisoprodol	9.90	261.17	176.12	8	24
		261.17	55.04	26	24
Cocaine	6.42	304.10	182.04	18	35
		304.10	81.97	28	35
Codeine	3.57	300.00	165.00	38	49
		300.00	215.03	26	49
Cyclobenzaprine	9.54	276.14	84.14	26	40
		276.14	58.12	20	40
Desalkylflurazepam	10.03	289.02	140.05	32	52
		289.02	226.13	30	52
Diazepam	10.32	285.26	154.06	26	48
		285.26	193.15	30	48
EDDP	8.51	278.28	234.11	30	56
		278.28	249.07	24	56
Fentanyl	8.17	337.19	105.13	40	46
		337.19	188.23	24	46
Flunitrazepam	9.43	314.05	268.21	28	48

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	Cone (V)
		314.05	239.21	40	48
Flurazepam	8.63	388.18	315.16	26	40
		388.18	100.13	28	40
Hydrocodone	4.06	300.10	198.97	28	53
		300.10	127.95	52	53
Hydromorphone	2.00	286.10	184.97	30	55
		286.10	157.01	44	55
Ketamine	5.90	238.06	125.03	28	32
		238.06	220.16	16	32
Lorazepam	9.90	321.04	275.00	20	38
		321.04	229.03	28	38
MDMA	4.34	194.02	163.10	12	26
		194.02	105.09	20	26
Meperidine	6.94	248.13	70.03	32	46
		248.13	174.16	24	46
Meprobamate	7.51	219.28	158.17	8	20
		219.28	55.06	24	20
Methadone	9.82	310.48	265.27	16	8
		310.48	105.08	28	8
Methamphetamine	4.22	150.26	91.05	18	18
		150.26	119.09	10	18
Methylphenidate	6.56	234.11	84.13	22	34
		234.11	56.04	50	34
Midazolam	9.17	326.05	291.12	28	54
		326.05	249.25	40	54
Morphine	1.34	286.11	165.15	38	52
		286.11	152.22	66	52
Naloxone	3.57	328.18	310.23	20	40
		328.18	212.17	42	40
Naltrexone	4.09	342.20	324.18	22	42
		342.20	55.06	44	42
Norbuprenorphine	7.90	414.29	83.09	50	64
		414.29	57.14	48	64
Norcarfentanil	6.77	291.27	113.09	28	28
		291.27	231.22	16	28
Nordiazepam	10.20	271.03	139.92	28	49
		271.03	90.95	36	49
Norfentanyl	6.05	233.36	84.09	18	30
		233.36	55.32	34	30
Norhydrocodone	4.11	286.08	199.01	28	38

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	Cone (V)
		286.08	127.94	52	38
Normeperidine	7.12	234.05	160.16	18	36
		234.05	42.06	32	36
Noroxycodone	3.89	302.14	186.98	22	40
		302.14	226.97	30	40
O-Desmethyltramadol	4.58	250.14	58.06	18	26
		250.14	42.22	60	26
Oxazepam	9.81	286.97	241.00	20	35
		286.97	103.94	34	35
Oxycodone	3.88	316.16	241.07	28	35
		316.16	256.03	24	35
Oxymorphone	1.58	302.10	226.99	26	43
		302.10	198.05	46	43
PCP	7.71	244.17	86.14	10	22
		244.17	91.09	30	22
Phentermine	5.15	149.96	91.05	22	18
		149.96	133.14	10	18
Propoxyphene	9.66	340.19	58.12	16	20
		340.19	266.26	8	20
Pseudoephedrine	3.42	166.26	148.11	14	22
		166.26	117.08	20	22
Sufentanil	9.27	387.24	238.16	20	36
		387.24	111.05	40	36
Δ^9-THC	11.16	315.43	123.10	32	36
		315.43	193.22	24	36
11-nor-9-carboxy-Δ^9-THC	10.84	345.41	327.32	16	38
		345.41	299.29	20	38
Tapentadol	6.63	222.20	106.94	26	30
		222.20	120.93	22	30
Temazepam	10.06	301.03	254.94	20	35
		301.03	176.97	36	35
Tramadol	6.40	264.16	58.12	18	28
		264.16	264.16	10	28
Trazodone	7.61	372.18	176.14	26	46
		372.18	148.12	38	46
Triazolam	9.98	343.01	308.15	28	54
		343.01	239.04	48	54
Zolpidem	7.29	308.14	235.24	40	54
		308.14	92.13	58	54

Table 9. Retention times, MRM transitions, and optimized mass spectrometer parameters for the deuterated internal standards.

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	Cone (V)
7-Aminoclonazepam-D6	5.87	290.16	121.00	32	50
7-Aminoflunitrazepam-D7	6.83	291.20	138.08	30	50
Alpha-Hydroxytriazolam-D4	9.51	363.04	176.09	30	52
Amphetamine-D5	3.76	141.03	124.15	8	24
Benzoylecgonine-D3	5.38	293.14	171.14	22	38
Buprenorphine-D4	9.15	472.35	59.01	50	67
Carisoprodol-D7	9.88	268.40	183.25	10	18
Cocaine-D3	6.42	307.10	185.04	20	39
Codeine-D6	3.53	306.20	61.11	34	54
Cyclobenzaprine-D3	9.54	279.18	216.03	26	40
Diazepam-D5	10.31	290.29	198.17	32	42
Fentanyl-D5	8.14	342.29	104.98	38	49
Flunitrazepam-D7	9.38	321.06	275.22	30	50
Hydromorphone-D3	1.97	289.16	184.98	30	57
Meperidine-D4	6.92	252.16	224.25	22	42
Methadone-D3	9.80	313.43	105.09	26	44
Methamphetamine-D5	4.19	155.05	91.94	18	26
Methylphenidate-D9	6.52	243.17	93.18	24	32
Morphine-D6	1.32	292.12	152.29	64	52
Nordiazepam-D5	10.19	276.16	140.16	24	61
Normeperidine-D4	7.11	238.08	164.19	18	38
Oxycodone-D6	3.85	322.13	304.31	20	42
PCP-D5	7.67	249.19	86.15	24	24
Propoxyphene-D5	9.63	345.21	58.12	14	20
Pseudoephedrine-D3	3.40	169.26	151.18	12	20
11-nor-9-carboxy-Δ^9-THC-D3	10.83	348.36	330.28	16	30

3.5.2 Shimadzu 8040 UFLC-MS/MS

Due to inconsistencies observed during analysis using the Waters UPLC-MS/MS, the second extraction was also analyzed on a Shimadzu 8040 UFLC-MS/MS, seen in Figure 4 (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using a Restek Raptor Biphenyl 2.7 µm column (50 x 2.1 mm) with a Raptor Biphenyl 2.7 µm guard cartridge (5 x 3.0 mm) attached to it (Restek Corporation, Bellefonte, PA). Mobile phase A consisted of 2

mM ammonium formate and 0.1% formic acid in water, while mobile phase B consisted of 2 mM ammonium formate and 0.1% formic acid in methanol. The LC pumps were held at a flow rate of 0.350 mL/min during analysis and raised to 0.500 mL/min for 1 minute between injections. The column oven was set to 30 °C during analysis and sample injections were set at 10 µL. This LC-MS/MS method was developed and validated by OSU-FTTL for clinical urine analysis.

The gradient program used for chromatographic separation of analytes began at a concentration of 10% mobile phase B and was increased to 35% over 1.40 min. The concentration of mobile phase B was further increased to 100% over 1.50 min and held at this concentration for another 1.00 min. The concentration was then returned to 10% in 0.01 min and maintained at this concentration until the next injection, for a total run time of 5 minutes.

Figure 4. A picture of the Shimadzu 8040 UFLC-MS/MS system used to analyze the wastewater samples in this study. On the left are the UFLC pumps, autosampler, communications bus module, UV-VIS detector, and column oven. On the right is the tandem mass spectrometer.

The MRM ion transitions used for analyte identification and quantification are summarized in Table 10 for the compounds of interest and Table 11 for the deuterated internal

standards. Compound identity was confirmed by use of the same parameters as the Waters Acquity Classic, described in section 3.5.1.

Table 10. Retention times, MRM transitions, and optimized mass spectrometer parameters used to analyze for compounds of interest on the Shimadzu 8040 UFLC-MS/MS. For each compound, the quantitative MRM transition is on the top row and the qualitative MRM transition is on the second row.

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
6-MAM	2.38	328.20	165.05	-10	-40	-30
		328.20	58.10	-10	-29	-20
7-Aminoclonazepam	2.98	286.00	121.10	-45	-32	-45
		286.00	222.10	-45	-24	-45
Alpha-Hydroxyalprazolam	3.49	324.90	297.05	-16	-27	-32
		324.90	216.00	-16	-42	-42
Alprazolam	3.62	309.30	281.00	-20	-27	-30
		309.30	205.00	-20	-45	-38
Amphetamine	1.88	136.00	91.10	-20	-17	-15
		136.00	119.15	-20	-14	-45
Benzoylecgonine	2.89	289.95	168.05	-14	-19	-30
		289.95	104.95	-20	-29	-18
Buprenorphine	3.12	468.10	55.10	-30	-55	-20
		468.30	84.05	-30	-49	-30
Carisoprodol	3.15	261.00	176.00	-30	-8	-18
		261.20	55.05	-28	-30	-20
Clonazepam	3.43	316.20	270.00	-50	-35	-30
		316.20	214.00	-50	-35	-45
Codeine	2.35	299.90	165.00	-35	-43	-30
		299.90	215.00	-35	-28	-20
Desipramine	3.25	267.00	44.10	-30	-35	-46
		267.00	72.15	-42	-25	-28
Diazepam	3.71	284.90	153.95	-36	-28	-28
		284.90	193.00	-46	-34	-34
EDDP	3.24	278.10	234.00	-15	-32	-45
		278.10	249.05	-35	-23	-25
Fentanyl	3.13	337.25	188.10	-22	-24	-18
		337.25	105.10	-22	-39	-38
Hydrocodone	2.58	299.90	199.00	-35	-40	-35

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
		300.30	171.10	-50	-40	-30
Hydromorphone	1.79	285.90	185.00	-32	-32	-34
		285.90	157.00	-32	-42	-26
JWH-018 Metabolite	3.70	358.00	154.95	-18	-25	-28
		358.00	126.95	-18	-52	-48
JWH-073 Metabolite	3.64	343.90	154.95	-40	-35	-28
		344.40	126.95	-12	-40	-48
Ketamine	2.89	238.20	125.00	-40	-27	-45
		238.20	207.10	-40	-14	-20
Lorazepam	3.36	320.80	274.90	-40	-23	-50
		320.80	229.00	-40	-30	-45
MDMA	2.47	194.05	163.10	-30	-14	-30
		194.05	105.05	-30	-26	-40
Meperidine	2.90	247.80	174.00	-44	-19	-32
		247.80	70.05	-40	-29	-26
Meprobamate	2.92	218.90	55.00	-24	-24	-20
		218.90	97.05	-24	-15	-36
Methadone	3.37	309.85	265.10	-40	-25	-28
		309.85	105.00	-36	-28	-38
Methamphetamine	2.20	149.75	91.00	-16	-10	-16
		149.75	119.05	-16	-15	-44
Morphine	1.57	286.10	165.00	-45	-42	-30
		286.10	155.10	-45	-35	-15
Norbuprenorphine	2.99	414.30	83.10	-25	-54	-15
		414.30	101.20	-25	-40	-40
Nordiazepam	3.52	271.20	139.90	-18	-29	-24
		271.20	208.10	-18	-30	-40
Norfentanyl	2.71	233.10	84.05	-40	-19	-30
		233.10	55.05	-15	-35	-20
Norhydrocodone	2.38	285.90	198.95	-32	-30	-36
		286.00	127.95	-14	-55	-46
Normeperidine	2.89	233.80	160.00	-26	-16	-28
		233.90	42.10	-26	-32	-14
Noroxycodone	2.29	302.10	187.00	-20	-26	-35
		302.10	227.00	-20	-31	-45
Nortriptyline	3.28	263.85	233.05	-50	-14	-24
		263.85	91.10	-50	-24	-36
Oxazepam	3.42	287.20	241.00	-50	-23	-25
		287.20	104.00	-15	-34	-40
Oxycodone	2.54	315.70	298.15	-35	-19	-30

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
		315.90	241.10	-40	-31	-50
Oxymorphone	1.64	301.90	227.00	-34	-30	-40
		301.90	198.00	-34	-48	-36
PCP	3.16	243.85	86.05	-44	-11	-14
		243.85	158.95	-44	-13	-28
Propoxyphene	3.15	340.40	266.20	-10	-17	-30
		266.30	58.00	-45	-14	-20
Tapentadol	2.77	222.10	107.05	-30	-27	-40
		222.10	121.05	-35	-21	-20
Temazepam	3.59	301.10	255.00	-20	-40	-25
		301.20	176.90	-20	-40	-30
THCA	3.68	345.10	299.10	-20	-20	-20
		345.10	192.90	-40	-27	-35
Tramadol	2.82	263.60	264.10	-30	-7	-30
		264.20	58.05	-10	-10	-20

Table 11. Retention times, MRM transitions, and optimized mass spectrometer parameters for the deuterated internal standards on the Shimadzu 8040 UFLC-MS/MS. For each compound, the quantitative MRM transition is on the top row and the qualitative MRM transition is on the second row.

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
7-Aminoclonazepam-D4	2.97	289.90	121.00	-34	-31	-46
		289.90	226.00	-34	-26	-42
Amphetamine-D5	1.85	140.80	93.00	-15	-19	-35
		140.80	66.00	-15	-40	-25
Benzoylecgonine-D3	2.89	292.80	171.05	-38	-19	-30
		292.80	105.00	-38	-33	-40
Codeine-D6	2.33	305.90	165.10	-40	-45	-30
		306.10	218.10	-15	-26	-45
Desipramine-D3	3.25	269.80	75.10	-32	-16	-28
		269.80	47.05	-32	-40	-50
Diazepam-D5	3.71	289.90	153.95	-50	-29	-28
		289.90	227.10	-50	-28	-24
Fentanyl-D5	3.13	341.85	105.10	-46	-42	-38
		341.85	137.10	-46	-35	-50
Hydromorphone-D3	1.79	288.90	184.95	-50	-31	-34
		288.90	156.95	-50	-44	-28
JWH-018 Metabolite-D5	3.69	363.60	155.00	-26	-24	-28

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
		363.60	127.00	-26	-52	-48
Meperidine-D4	2.89	251.80	224.10	-42	-22	-22
		251.80	178.05	-42	-20	-32
Methadone-D3	3.33	312.85	268.15	-48	-15	-28
		312.85	104.95	-48	-30	-42
Methamphetamine-D5	2.18	154.95	92.10	-16	-20	-34
		154.90	66.10	-16	-42	-24
Morphine-D6	1.55	292.20	152.00	-20	-54	-50
		292.20	181.10	-20	-38	-35
Normeperidine-D4	2.89	238.00	164.10	-12	-16	-16
		238.00	42.05	-12	-35	-44
Nortryptyline-D3	3.27	266.95	233.10	-50	-15	-24
		266.95	91.00	-50	-24	-34
Oxycodone-D6	2.53	321.90	304.15	-38	-20	-32
		321.90	247.10	-38	-33	-42
PCP-D5	3.15	248.85	86.10	-30	-12	-34
		248.85	96.00	-30	-32	-34
Propoxyphene-D5	3.15	345.10	58.15	-22	-23	-22
		345.10	271.20	-22	-10	-18
THC-D3	3.70	348.10	331.00	-20	-8	-35
		348.10	313.00	-50	-12	-30

3.5.3 LC-MS/MS Lower Limit of Quantitation

The lower limit of quantitation (LLOQ) for each compound of interest varied between analyses when using Waters UPLC-MS/MS method. The first set of wastewater samples, calibration curve, QCs, and blank was only analyzed on the Waters. The second set of extracted samples, calibrators, QCs, and blank was analyzed on both the Waters and Shimadzu. The LLOQ concentrations for each analysis are given in Table 12.

Table 12. LLOQ Concentrations for each analyte of interest across the 3 different wastewater analyses.

Analyte Name	1st Waters Analysis LLOQ (ng/mL)	2nd Waters Analysis LLOQ (ng/mL)	Shimadzu Analysis LLOQ (ng/mL)
6-MAM	0.05	0.05	0.05
7-Aminoclonazepam	0.05	0.05	0.05
7-Aminoflunitrazepam	0.05	0.05	N/A

Analyte Name	1st Waters Analysis LLOQ (ng/mL)	2nd Waters Analysis LLOQ (ng/mL)	Shimadzu Analysis LLOQ (ng/mL)
α-Hydroxyalprazolam	0.05	0.05	0.05
α-Hydroxytriazolam	0.05	0.05	N/A
Alprazolam	0.05	0.05	0.05
Amphetamine	0.5	0.05	0.05
Benzoylecgonine	0.05	0.05	0.05
Buprenorphine	0.05	0.75	0.75
Carfentanil	0.05	0.75	N/A
Carisoprodol	0.5	0.05	0.05
Cocaine	0.05	0.15*	N/A
Codeine	0.05	0.05	0.05
Cyclobenzaprine	0.5	1.25	N/A
Desalkylflurazepam	0.5	0.05	N/A
Diazepam	0.05	0.05	0.05
EDDP	0.05	0.75	0.75
Fentanyl	0.05	0.5	0.5
Flunitrazepam	0.05	0.05	N/A
Flurazepam	0.05	1.25	N/A
Hydrocodone	0.05	0.05	0.05
Hydromorphone	0.05	0.5	0.05
Ketamine	0.05	0.05	0.05
Lorazepam	0.05	0.05	0.5
MDMA	0.05	0.05	0.05
Meperidine	0.05	0.5	0.5
Meprobamate	0.5	0.5	0.5
Methadone	0.5	2.5	2.5
Methamphetamine	0.5	0.15*	0.5*
Methylphenidate	0.05	0.05	N/A
Midazolam	0.05	0.5	N/A
Morphine	0.05	0.05	0.05
Naloxone	0.05	0.05	N/A
Naltrexone	0.05	0.05	N/A
Norbuprenorphine	0.05	0.05	0.5
Norcarfentanil	0.05	0.05	N/A
Nordiazepam	0.05	0.05	0.05
Norfentanyl	0.05	0.05	0.05
Norhydrocodone	0.05	0.05	0.05
Normeperidine	0.05	0.5	0.5
Noroxycodone	0.05	0.05	0.05
O-Desmethyltramadol	0.5	0.05	N/A

Analyte Name	1st Waters Analysis LLOQ (ng/mL)	2nd Waters Analysis LLOQ (ng/mL)	Shimadzu Analysis LLOQ (ng/mL)
Oxazepam	0.05	0.05	0.5
Oxycodone	0.5	0.05	0.05
Oxymorphone	0.05	0.05	0.05
PCP	0.05	0.5	0.5
Phentermine	0.05	0.05	N/A
Propoxyphene	0.05	0.5	0.5
Pseudoephedrine	0.05	0.05	N/A
Sufentanil	0.05	0.5	N/A
Tapentadol	0.05	0.05	0.5
Temazepam	0.05	0.05	0.05
Tramadol	0.05	0.05	0.5
Trazodone	0.05	0.5	N/A
Triazolam	0.05	0.5	N/A
Zolpidem	0.05	0.5	N/A

* LLOQ between 1c (0.05 ng/mL) and 10c (0.5 ng/mL) calibrators due to analyte contamination observed in the blank.

3.6 Summary

Wastewater samples collected by UF were extracted via SPE and analyzed at OSU-FTTL using two different LC-MS/MS systems. If a compound of interest was able to be quantified above the lowest calibrator, lower limit of quantitation (LLOQ), and was within a 20% range of the averaged MRM ratio, it was considered to be present in the sample.

CHAPTER IV

RESULTS

4.1 LC-MS/MS Results

Concentrations of the compounds of interest were obtained using the methods described in Chapter III. The results each LC-MS/MS analysis are summarized in their own subsections for clarity.

4.1.1 Initial Extraction Analysis on the Waters UPLC-MS/MS

The results of the 4 background samples obtained on September 5, 2018 are given in Table 13. The results of the first batch of samples extracted and analyzed on the Waters system are given in Table 14.

Table 13. Concentrations of compounds observed in the 4 background waste water samples collected before the UF game. Compounds that were not observed as being present in any of the samples were removed from the table for clarity. Concentrations are reported in ng/mL.

Compound Name	Background 1	Background 2	Background 3	Background 4
Benzoylecgonine	0.2	0.3	-	0.3
Cocaine	-	0.1	-	-
Ketamine	-	-	-	0.1
Morphine	-	0.1	-	0.1
Phentermine	-	-	-	0.2
Pseudoephedrine	-	0.4	0.2	1.2
Tramadol	-	0.1	-	0.2
Trazodone	-	-	0.1	0.1

Table 14. Concentrations of compounds observed in the initially extracted 33 wastewater samples collected during the game. Compounds that were not observed as being present in any of the samples were removed from the table for clarity. Concentrations are reported in ng/mL.

Sample #	7-Aminoclonazepam	Amphetamine	Benzoylecgonine	Cocaine
1	*	††	0.8	0.3
2	-	††	1.9	0.4
3	-	††	2.0	1.9
4	-	0.7	0.8	0.7
5	-	††	6.3	1.8
6	-	1.0	0.8	0.4
7	-	††	2.1	1.3
8	-	1.0	2.0	0.4
9	-	5.2	1.0	1.2
10	-	*	3.9	5.0
11	-	1.1	1.9	1.1
12	-	††	2.5	2.2
13	-	0.7	1.3	1.2
14	-	3.5	2.0	1.0
15	-	4.6	7.2	5.1
16	-	4.2	20	0.9
17	-	0.8	1.8	1.2
18	-	1.5	2.1	0.9
19	-	0.8	4.0	1.1
20	-	4.0	5.9	0.8
21	-	2.4	3.7	2.2
22	-	2.1	0.5	0.6
23	-	5.2	0.5	0.5
24	-	††	0.9	2.3
25	-	††	3.1	10
26	-	1.9	0.1	0.1
27	†	†	†	†
28	-	2.3	0.1	0.1
29	-	††	0.6	0.2
30	-	††	10	2.6
31	-	3.7	*	-
32	-	††	0.2	0.1
33	-	††	0.3	0.7

* Detected below the lower limit of quantitation, ** Detected outside the allowed 20% ratio range, *** Detected below the lower limit of quantitation and outside of 20% ratio range, † Sample 27 Omitted due to suspected contamination, †† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Codeine	Cyclobenzaprine	EDDP	Hydrocodone
1	-	-	-	0.2
2	-	-	-	0.2
3	-	0.6	-	0.1

4	-	-	-	*
5	*	-	-	-
6	-	-	-	0.1
7	-	-	-	-
8	-	-	0.3	0.1
9	0.3	-	-	0.1
10	-	-	-	-
11	-	-	-	0.1
12	-	-	-	*
13	-	-	0.1	-
14	*	-	-	0.1
15	-	-	-	-
16	-	-	-	-
17	-	-	-	0.1
18	-	-	-	-
19	***	-	-	-
20	***	-	-	0.6
21	-	-	-	0.6
22	-	-	-	0.2
23	-	-	-	0.3
24	-	-	-	0.1
25	0.2	-	-	*
26	-	-	0.1	0.2
27	†	†	†	†
28	-	-	-	-
29	0.2	-	-	0.1
30	-	-	-	0.2
31	-	-	-	0.1
32	-	-	-	0.2
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Hydromorphone	Ketamine	MDMA	Methadone
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	*	0.3	-	1.1
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	0.1	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	*	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	0.3	2.6	-
24	-	-	-	-
25	-	-	-	-
26	-	-	0.1	-
27	†	†	†	†
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Methamphetamine	Methylphenidate	Morphine	Norhydrocodone
1	-	-	-	0.3
2	-	-	-	0.1
3	-	-	0.3	0.1
4	-	-	-	-
5	*	-	-	-
6	1.1	*	-	0.1
7	-	-	-	-
8	-	-	*	0.1
9	-	-	-	-
10	-	0.1	-	-
11	*	-	-	0.1**
12	-	-	-	-
13	-	-	-	-
14	*	-	-	0.2
15	-	-	-	-
16	-	-	-	-
17	-	-	-	0.1
18	-	-	-	-
19	-	-	-	-
20	-	-	0.1	0.5
21	-	-	-	0.3
22	-	-	-	0.1
23	-	-	0.1	0.3
24	-	-	-	0.1
25	-	-	-	-
26	-	-	-	0.2
27	†	†	†	†
28	-	-	-	-
29	-	-	-	0.1
30	-	-	-	0.1
31	-	-	-	0.1
32	-	-	-	0.1
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Noroxycodone	O-Desmethyltramadol	Oxycodone	Oxymorphone
1	0.1	-	*	-
2	0.3	-	*	*
3	0.2	0.9	*	*
4	-	-	-	-
5	0.1	-	*	*
6	0.1	0.7	*	-
7	-	0.3	*	-
8	-	-	-	-
9	-	-	*	-
10	-	-	-	-
11	-	-	-	-
12	0.2	-	*	-
13	2.6	4.5	*	-
14	0.3	1.7	*	*
15	0.5	0.6	*	-
16	-	-	-	-
17	1.0	-	*	*
18	0.1**	-	*	-
19	-	-	-	-
20	0.7	-	*	*
21	0.3	-	*	-
22	-	-	-	-
23	0.2	-	*	-
24	0.1	0.6**	*	-
25	-	1.4	-	-
26	0.1	-	-	-
27	†	†	†	†
28	-	-	-	-
29	0.1	0.6	*	-
30	0.2	1.7	*	-
31	-	-	-	-
32	*	-	*	-
33	0.3	-	***	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Phentermine	Pseudoephedrine	Tapentadol	Temazepam
1	0.5	10	-	-
2	0.7	24	-	-
3	4.8	9.6	1.5	0.2
4	0.3	2.4	-	-
5	2.2	2.5	-	-
6	0.3	4.5	-	-
7	1.3	6.0	-	-
8	0.1	3.1	-	***
9	2.5	19	0.4	*
10	0.9	8.0	-	-
11	*	9.1	-	-
12	0.2	14	-	-
13	-	7.2	-	-
14	1.1	9.3	-	*
15	3.5	19	-	-
16	4.9	10	-	-
17	-	8.8	-	*
18	4.6	4.5	-	-
19	1.5	36	-	-
20	1.8	14	-	***
21	0.1	27	-	-
22	1.8	1.4	-	-
23	3.6	17	-	*
24	1.4	74	-	-
25	0.1	9.0	-	-
26	0.6	15	-	-
27	†	†	†	†
28	2.2	0.6	-	-
29	1.5	9.7	-	-
30	0.4	4.5	-	-
31	0.3	32	-	-
32	0.7	6.3	-	-
33	1.2	7.3	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

Table 14. (continued)

Sample #	Tramadol	Trazodone
1	-	-
2	1.0	-
3	2.9	0.1
4	-	-
5	-	0.2
6	3.5	-
7	1.5	-
8	0.2	-
9	-	-
10	2.5	0.2
11	2.1	0.1
12	0.1	0.3
13	14	-
14	4.1	-
15	2.2	0.1
16	0.4	-
17	0.2	-
18	0.6	-
19	0.3	-
20	0.7	0.1
21	0.5	-
22	-	0.1
23	0.3	0.1
24	1.9	0.1
25	6.0	-
26	-	-
27	†	†
28	0.1	-
29	2.6	-
30	7.4	-
31	0.1	-
32	0.2	-
33	1.0	0.1

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
† Sample 27 Omitted due to suspected contamination
†† No quantitative data available due to nearby unknown interfering peak resulting in a ratio outside of the 20% range

4.1.2 Second Waters UPLC-MS/MS Extraction Results

The results of the second batch of samples extracted and analyzed on the Waters are given in Table 15. Note that the 4 background samples were not re-extracted and analyzed a second time due to insufficient volume.

Table 15. Concentrations of compounds observed in the second extraction of the 33 wastewater samples collected during the UF home football game. Compounds that were not observed as being present in any of the samples were removed from the table for clarity. Concentrations are reported in ng/mL.

Sample #	7-Aminoclonazepam	Amphetamine	Benzoylecgonine	Cocaine
1	0.1	0.4	1.2	0.2
2	-	0.4	1.8	0.4
3	-	0.5	2.0	1.3
4	-	0.5	0.6	0.6
5	-	1.2	6.2	0.8
6	-	1.0	1.0	0.6
7	-	0.7	2.2	1.8
8	-	1.0	2.0	0.3
9	-	4.8	1.6	2.0
10	-	0.2	3.3	5.0
11	-	1.4	2.6	1.4
12	-	1.1	1.2	1.6
13	-	0.7	1.1	1.5
14	-	2.8	2.6	0.9
15	-	1.7	2.9	2.9
16	-	2.7	14	0.8
17	-	0.8	1.7	1.1
18	-	1.0	1.7	0.9
19	-	0.9	4.0	1.3
20	-	2.6	4.9	0.7
21	-	1.2	2.3	1.9
22	-	2.2	0.6	0.8
23	-	3.5	0.4	0.4
24	-	0.3	0.8	2.7
25	-	0.3	2.6	11
26	-	1.0	0.1	*
27	-	2.3	5.2	4.9
28	-	2.4	0.1	0.2
29	-	0.2	0.6	0.2
30	-	0.5	10	3.5
31	-	2.7	0.1	-
32	-	2.6	0.3	*
33	-	0.5	0.4	1.0

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range

*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Codeine	Cyclobenzaprine	EDDP	Hydrocodone
1	-	-	-	0.3
2	-	-	-	0.2
3	-	*	-	0.1
4	-	-	-	0.1
5	0.1	-	-	-
6	-	-	-	0.2
7	-	-	-	-
8	-	-	1.5	0.2
9	0.4	-	-	0.2
10	-	-	-	-
11	-	-	-	0.1
12	-	-	-	0.1
13	-	-	*	-
14	0.1	-	-	0.2
15	-	-	-	-
16	-	-	-	-
17	-	-	-	0.1
18	-	-	-	-
19	0.1	-	-	-
20	0.1	-	-	0.7
21	-	-	-	0.7
22	-	-	-	0.3
23	-	-	-	0.3
24	-	-	-	0.2
25	0.2	-	-	***
26	-	-	*	0.2
27	-	-	-	-
28	-	-	-	-
29	0.2	-	-	0.1
30	-	-	-	0.2
31	-	-	-	0.1
32	-	-	-	0.3
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Hydromorphone	Ketamine	MDMA	Methadone
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-

8	*	0.4	-	2.5
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	0.1	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	*	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	0.3	1.3	-
24	-	-	-	-
25	-	-	-	-
26	-	-	0.1	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Methamphetamine	Methylphenidate	Morphine	Norhydrocodone
1	-	-	-	0.4
2	-	-	-	0.1
3	-	-	0.4	0.1
4	-	-	-	-
5	0.2	-	-	-
6	1.2	0.1	-	0.1
7	-	-	-	-
8	-	-	0.1	0.1
9	-	-	-	-
10	-	0.1	-	-
11	0.6	-	-	0.1
12	-	-	-	-
13	-	-	-	-
14	0.2	-	0.1	0.2
15	-	-	-	-
16	-	-	-	-

17	-	-	-	0.1
18	-	-	-	-
19	-	-	-	-
20	-	-	1.3	0.4
21	-	-	-	0.2
22	-	-	-	0.1
23	-	-	0.2	0.2
24	-	-	-	0.1
25	-	-	-	-
26	-	-	-	0.1
27	-	-	-	-
28	-	-	-	-
29	-	-	-	0.1
30	-	-	-	0.1
31	-	-	-	0.1
32	-	-	-	0.1
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Noroxycodone	O-Desmethyltramadol	Oxycodone	Oxymorphone
1	0.2	-	0.1	-
2	0.3	-	0.3	0.1
3	0.2	0.6	0.2	0.1
4	-	-	-	-
5	0.1**	-	0.1	0.1
6	0.1	0.7	0.1	-
7	-	0.3	*	-
8	-	-	-	-
9	-	-	*	-
10	-	-	-	-
11	-	-	-	-
12	0.1	-	0.1	-
13	2.5	2.7	0.2	-
14	0.4	2.8	0.3	0.1
15	0.3	***	0.3	-
16	-	-	-	-
17	1.0	-	0.4	0.1
18	0.2	-	0.5	-
19	-	-	-	-
20	0.7	-	0.4	0.3
21	0.3	-	0.2	-
22	-	-	-	-
23	0.2	-	0.2	-
24	0.1**	0.6	0.1	-
25	-	0.9	-	-
26	***	-	-	-
27	-	-	-	-
28	-	-	-	-
29	0.2	0.4	0.1	-
30	0.3	1.1	0.2	-
31	-	-	-	-
32	0.1	-	*	-
33	0.6	-	0.3	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Phentermine	Pseudoephedrine	Tapentadol	Temazepam
1	1.0	15	-	-
2	0.7	21	-	-
3	4.7	7.5	1.3	0.3
4	0.3	1.6	-	-
5	2.3	2.1	-	-
6	0.5	5.4	-	-
7	1.4	6.1	-	-
8	0.1	2.9	-	0.1
9	4.1	27	0.5	*
10	0.9	6.7	-	-
11	0.1	13	-	-
12	0.1	6.2	-	-
13	-	5.7	-	-
14	1.5	10	-	0.1**
15	1.5	7.2	-	-
16	3.7	7.2	-	-
17	-	8.0	-	0.4
18	4.2	3.7	-	-
19	1.6	35	-	-
20	1.6	12	-	0.1
21	0.1	16	-	-
22	2.3	1.8	-	-
23	3.2	13	-	0.2**
24	1.5	62	-	-
25	0.1	7.6	-	-
26	0.3	8.1	-	-
27	0.1	6.5	-	-
28	3.0	0.8	-	-
29	1.7	10	-	-
30	0.5	5.3	-	-
31	0.3	31	-	-
32	1.0	8.6	-	-
33	1.9	12	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

Table 15. (continued)

Sample #	Tramadol	Trazodone
1	-	-
2	1.1**	-
3	2.8**	*
4	-	-
5	-	*
6	4.6	-
7	1.9	-
8	0.3**	-
9	-	-
10	2.4	*
11	3.4	*
12	0.1**	0.5
13	15	-
14	5.4	-
15	1.0	*
16	0.3**	-
17	0.3	-
18	0.7	-
19	0.3**	-
20	0.6	*
21	0.4**	-
22	-	*
23	0.3**	*
24	2.2	*
25	5.7	-
26	-	-
27	0.4	-
28	0.2**	-
29	2.9	-
30	9.2	-
31	0.2**	-
32	0.4	-
33	1.4	*

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range

4.1.3 Shimadzu 8040 UFLC-MS/MS Data

The results of the second analysis of the wastewater samples performed on the Shimadzu, are given in Table 16.

Table 16. Concentrations of compounds obtained from the extracted wastewater samples on the Shimadzu UFLC-MS/MS. Compounds that were not observed as being present in any of the samples were removed from the table for clarity. Concentrations are reported in ng/mL.

Sample #	7-Aminoclonazepam	Amphetamine	Benzoylecgonine	Cocaine
1	0.1**	0.3	1.3	N/A
2	-	0.3	1.8	N/A
3	-	0.4	1.8	N/A
4	-	0.4	0.5	N/A
5	-	1.0	6.1	N/A
6	-	0.9	0.9	N/A
7	-	0.6	2.1	N/A
8	-	0.8	1.9	N/A
9	-	6.5	1.4	N/A
10	-	0.2	3.5	N/A
11	-	1.3	2.6	N/A
12	-	1.0	1.2	N/A
13	-	0.5	1.1	N/A
14	-	3.1	2.6	N/A
15	-	1.5	2.8	N/A
16	-	2.3	14	N/A
17	-	0.6	1.7	N/A
18	-	0.9	1.5	N/A
19	-	0.8	4.0	N/A
20	-	2.2	4.6	N/A
21	-	1.1	2.2	N/A
22	-	1.7	0.5	N/A
23	-	3.1	0.4	N/A
24	-	0.3	0.8	N/A
25	-	0.3	2.4	N/A
26	-	0.8	0.1	N/A
27	-	2.4	4.8	N/A
28	-	2.0	0.1	N/A
29	-	0.2	0.5	N/A
30	-	0.3	9.8	N/A
31	-	2.3	0.1	N/A
32	-	1.9	0.3	N/A
33	-	0.5	0.3	N/A

* Detected below the lower limit of quantitation

** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Codeine	Cyclobenzaprine	EDDP	Hydrocodone
1	-	N/A	-	0.4
2	-	N/A	-	0.3
3	-	N/A	-	0.1
4	-	N/A	-	0.1
5	***	N/A	-	-
6	-	N/A	-	0.3
7	-	N/A	-	-
8	-	N/A	1.3	0.2
9	0.4	N/A	-	0.2
10	-	N/A	-	-
11	-	N/A	-	0.2
12	-	N/A	-	0.1
13	-	N/A	*	-
14	0.1	N/A	-	0.1
15	-	N/A	-	-
16	-	N/A	-	-
17	-	N/A	-	0.1
18	-	N/A	-	-
19	0.1	N/A	-	-
20	0.1	N/A	-	0.8
21	-	N/A	-	1.0
22	-	N/A	-	0.4
23	-	N/A	-	0.5
24	-	N/A	-	0.2
25	0.2	N/A	-	0.1
26	-	N/A	*	0.3
27	-	N/A	-	-
28	-	N/A	-	-
29	0.2	N/A	-	0.2
30	-	N/A	-	0.4
31	-	N/A	-	0.2
32	-	N/A	-	0.4
33	-	N/A	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Hydromorphone	Ketamine	MDMA	Methadone
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	0.1	0.4	-	*
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	0.1	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	0.1	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	0.2	2.3	-
24	-	-	-	-
25	-	-	-	-
26	-	-	0.1	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Methamphetamine	Methylphenidate	Morphine	Norhydrocodone
1	-	N/A	-	0.2
2	-	N/A	-	0.1
3	-	N/A	0.5	*
4	-	N/A	-	-
5	0.2	N/A	-	-
6	1.7	N/A	-	0.1
7	-	N/A	-	-
8	-	N/A	0.1	*
9	-	N/A	-	-
10	-	N/A	-	-
11	0.8	N/A	-	***
12	-	N/A	-	-
13	-	N/A	-	-
14	0.2	N/A	0.1	0.1
15	-	N/A	-	-
16	-	N/A	-	-
17	-	N/A	-	***
18	-	N/A	-	-
19	-	N/A	-	-
20	-	N/A	1.2	0.1
21	-	N/A	-	0.1
22	-	N/A	-	*
23	-	N/A	0.2	0.1
24	-	N/A	-	*
25	-	N/A	-	-
26	-	N/A	-	*
27	-	N/A	-	-
28	-	N/A	-	-
29	-	N/A	-	*
30	-	N/A	-	*
31	-	N/A	-	*
32	-	N/A	-	*
33	-	N/A	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Noroxycodone	O-Desmethyltramadol	Oxycodone	Oxymorphone
1	0.1	N/A	0.2	-
2	0.2	N/A	0.3	0.1
3	0.1	N/A	0.2	0.1
4	-	N/A	-	-
5	0.1	N/A	0.1	0.1
6	0.1	N/A	0.1	-
7	-	N/A	0.1	-
8	-	N/A	-	-
9	-	N/A	0.1	-
10	-	N/A	-	-
11	-	N/A	-	-
12	0.1	N/A	0.1	-
13	1.1	N/A	0.2	-
14	0.2	N/A	0.4	0.2
15	0.2	N/A	0.3	-
16	-	N/A	-	-
17	0.4	N/A	0.5	0.1
18	*	N/A	0.6	-
19	-	N/A	-	-
20	0.5	N/A	0.4	0.5
21	0.1	N/A	0.2	-
22	-	N/A	-	-
23	0.1	N/A	0.2	-
24	*	N/A	0.1	-
25	-	N/A	-	-
26	*	N/A	-	-
27	-	N/A	-	-
28	-	N/A	-	-
29	*	N/A	0.2	-
30	0.1	N/A	0.3	-
31	-	N/A	-	-
32	*	N/A	0.1	-
33	0.3	N/A	0.4	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Phentermine	Pseudoephedrine	Tapentadol	Temazepam
1	N/A	N/A	-	-
2	N/A	N/A	-	-
3	N/A	N/A	1.6	0.6
4	N/A	N/A	-	-
5	N/A	N/A	-	-
6	N/A	N/A	-	-
7	N/A	N/A	-	-
8	N/A	N/A	-	0.1
9	N/A	N/A	0.8	0.1
10	N/A	N/A	-	-
11	N/A	N/A	-	-
12	N/A	N/A	-	-
13	N/A	N/A	-	-
14	N/A	N/A	-	0.1
15	N/A	N/A	-	-
16	N/A	N/A	-	-
17	N/A	N/A	-	0.6
18	N/A	N/A	-	-
19	N/A	N/A	-	-
20	N/A	N/A	-	0.2
21	N/A	N/A	-	-
22	N/A	N/A	-	-
23	N/A	N/A	-	0.3
24	N/A	N/A	-	-
25	N/A	N/A	-	-
26	N/A	N/A	-	-
27	N/A	N/A	-	-
28	N/A	N/A	-	-
29	N/A	N/A	-	-
30	N/A	N/A	-	-
31	N/A	N/A	-	-
32	N/A	N/A	-	-
33	N/A	N/A	-	-

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

Table 16. (continued)

Sample #	Tramadol	Trazodone
1	-	N/A
2	1.2	N/A
3	3.1	N/A
4	-	N/A
5	-	N/A
6	5.5	N/A
7	2.1	N/A
8	*	N/A
9	-	N/A
10	2.2	N/A
11	3.7	N/A
12	***	N/A
13	19****	N/A
14	6.2	N/A
15	1.2	N/A
16	0.5	N/A
17	*	N/A
18	0.9	N/A
19	0.5	N/A
20	0.8	N/A
21	0.6	N/A
22	-	N/A
23	*	N/A
24	2.7	N/A
25	5.2	N/A
26	-	N/A
27	0.6	N/A
28	*	N/A
29	3.4	N/A
30	11****	N/A
31	*	N/A
32	0.5	N/A
33	1.5	N/A

* Detected below the lower limit of quantitation
** Detected outside the allowed 20% ratio range
*** Detected below the lower limit of quantitation and outside of 20% ratio range
**** Detected above the upper limit of quantitation
N/A Analyte not screened for in the Shimadzu's validated urinalysis method

4.2 Summary

Final concentrations for each sample were reported based on the results obtained from all three (3) analyses. Compounds of interest present in the Shimadzu UFLC-MS/MS method were reported in Table 17 as observed. The reasoning behind this decision is the Shimadzu is method is validated for urine and has a history of regular, reliable performance screening clinical samples. Therefore, while the Shimadzu method is not validated for wastewater samples, the possibility of instrumental error on the Waters can be ruled out for the Shimadzu.

For drugs not present in the Shimadzu method, an average of the two concentrations obtained from the Waters UPLC-MS/MS analyses is also reported in Table 17. All final concentrations are considered semi-quantitative since neither the Waters nor Shimadzu analysis methods are validated for wastewater. Concentrations were rounded up to 2 significant figures. Figure 5 contains a breakdown of the number of samples containing at least one drug from each class.

Table 17. Final concentrations for each of the game day samples based on the three analysis. Analytes present in the Shimadzu method were reported as detected. Concentrations of analytes present only on the Waters were reported as an average of the two analyses. Compounds that were not observed as being present in any of the samples were removed from the table for clarity. Concentrations are reported in ng/mL.

Sample #	7-Aminoclonazepam	Amphetamine	Benzoylecgonine	Cocaine
1	0.1	0.3	1.3	0.3
2	-	0.3	1.8	0.4
3	-	0.4	1.8	1.6
4	-	0.4	0.5	0.7
5	-	1.0	6.1	1.3
6	-	0.9	0.9	0.5
7	-	0.5	2.1	1.5
8	-	0.8	1.9	0.4
9	-	6.5	1.4	1.6
10	-	0.2	3.5	5.0
11	-	1.3	2.6	1.3
12	-	1.0	1.2	1.9
13	-	0.5	1.1	1.3
14	-	3.1	2.6	0.9
15	-	1.4	2.8	4.0

Sample #	7-Aminoclonazepam	Amphetamine	Benzoylecgonine	Cocaine
16	-	2.3	14	0.8
17	-	0.6	1.7	1.1
18	-	0.9	1.5	0.9
19	-	0.8	4.0	1.2
20	-	2.2	4.6	0.8
21	-	1.1	2.2	2.0
22	-	1.7	0.5	0.7
23	-	3.1	0.4	0.5
24	-	0.3	0.8	2.5
25	-	0.2	2.4	11
26	-	0.8	0.1	0.1
27	-	2.4	4.8	4.9
28	-	2.0	0.1	0.1
29	-	0.2	0.5	0.2
30	-	0.3	9.8	3.1
31	-	2.3	0.1	-
32	-	1.9	0.3	0.1
33	-	0.5	0.3	0.9

Table 17. (continued)

Sample #	Codeine	Cyclobenzaprine	EDDP	Hydrocodone
1	-	-	-	0.4
2	-	-	-	0.3
3	-	0.6	-	0.1
4	-	-	-	0.1
5	0.1	-	-	-
6	-	-	-	0.3
7	-	-	-	-
8	-	-	1.3	0.2
9	0.4	-	-	0.2
10	-	-	-	-
11	-	-	-	0.2
12	-	-	-	0.1
13	-	-	0.1	-
14	0.1	-	-	0.1
15	-	-	-	-
16	-	-	-	-
17	-	-	-	0.1
18	-	-	-	-
19	0.1	-	-	-
20	0.1	-	-	0.8
21	-	-	-	1.0
22	-	-	-	0.4
23	-	-	-	0.5
24	-	-	-	0.2
25	0.2	-	-	0.1
26	-	-	0.1	0.3
27	-	-	-	-
28	-	-	-	-
29	0.2	-	-	0.2
30	-	-	-	0.4
31	-	-	-	0.2
32	-	-	-	0.4
33	-	-	-	-

Table 17. (continued)

Sample #	Hydromorphone	Ketamine	MDMA	Methadone
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	0.1	0.3	-	1.1
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	0.1	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	0.1	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	0.2	2.1	-
24	-	-	-	-
25	-	-	-	-
26	-	-	0.1	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-

Table 17. (continued)

Sample #	Methamphetamine	Methylphenidate	Morphine	Norhydrocodone
1	-	-	-	0.2
2	-	-	-	0.1
3	-	-	0.5	-
4	-	-	-	-
5	0.2	-	-	-
6	1.7	0.1	-	0.6
7	-	-	-	-
8	-	-	0.1	0.7
9	-	-	-	-
10	-	0.1	-	-
11	0.8	-	-	-
12	-	-	-	-
13	-	-	-	-
14	0.2	-	0.1	0.1
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	-	-	1.2	0.1
21	-	-	-	0.1
22	-	-	-	-
23	-	-	0.2	0.1
24	-	-	-	-
25	-	-	-	-
26	-	-	-	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-

Table 17. (continued)

Sample #	Noroxycodone	O-Desmethyltramadol	Oxycodone	Oxymorphone
1	0.1	-	0.2	-
2	0.2	-	0.3	0.1
3	0.1	0.7	0.2	0.1
4	-	-	-	-
5	0.1	-	0.1	0.1
6	0.1	0.7	0.1	-
7	-	0.3	0.1	-
8	-	-	-	-
9	-	-	0.1	-
10	-	-	-	-
11	-	-	-	-
12	0.1	-	0.1	-
13	1.1	3.6	0.2	-
14	0.2	2.2	0.4	0.2
15	0.2	0.6	0.3	-
16	-	-	-	-
17	0.4	-	0.5	0.1
18	0.2	-	0.6	-
19	-	-	-	-
20	0.5	-	0.4	0.5
21	0.1	-	0.2	-
22	-	-	-	-
23	0.1	-	0.2	-
24	-	0.6	0.1	-
25	-	1.1	-	-
26	-	-	-	-
27	-	-	-	-
28	-	-	-	-
29	0.1	0.5	0.2	-
30	0.1	1.4	0.3	-
31	-	-	-	-
32	0.1	-	0.1	-
33	0.3	-	0.4	-

Table 17. (continued)

Sample #	Phentermine	Pseudoephedrine	Tapentadol	Temazepam
1	0.8	12	-	-
2	0.7	23	-	-
3	4.7	8.6	1.6	0.6
4	0.3	2.0	-	-
5	2.3	2.3	-	-
6	0.4	4.9	-	-
7	1.3	6.0	-	-
8	0.1	3.0	-	0.1
9	3.3	23	0.8	0.1
10	0.9	7.4	-	-
11	0.1	11	-	-
12	0.1	10	-	-
13	-	6.4	-	-
14	1.3	10	-	0.1
15	2.5	13	-	-
16	4.3	8.7	-	-
17	-	8.4	-	0.6
18	4.4	4.1	-	-
19	1.5	35	-	-
20	1.7	13	-	0.2
21	0.1	22	-	-
22	2.0	1.6	-	-
23	3.4	15	-	0.3
24	1.4	68	-	-
25	0.1	8.4	-	-
26	0.5	12	-	-
27	0.1	6.5	-	-
28	2.6	0.7	-	-
29	1.6	9.9	-	-
30	0.5	4.9	-	-
31	0.3	32	-	-
32	0.9	7.4	-	-
33	1.6	9.8	-	-

Table 17. (continued)

Sample #	Tramadol	Trazadone
1	-	-
2	1.2	-
3	3.1	0.1
4	-	-
5	-	0.2
6	5.5	-
7	2.1	-
8	-	-
9	-	-
10	2.2	0.2
11	3.7	0.1
12	-	0.3
13	14	-
14	6.2	-
15	1.2	0.1
16	0.5	-
17	0.2	-
18	0.9	-
19	0.5	-
20	0.8	0.1
21	0.6	-
22	-	0.1
23	-	0.1
24	2.7	0.1
25	5.2	-
26	-	-
27	0.6	-
28	-	-
29	3.4	-
30	8.3	-
31	-	-
32	0.5	-
33	1.5	0.1

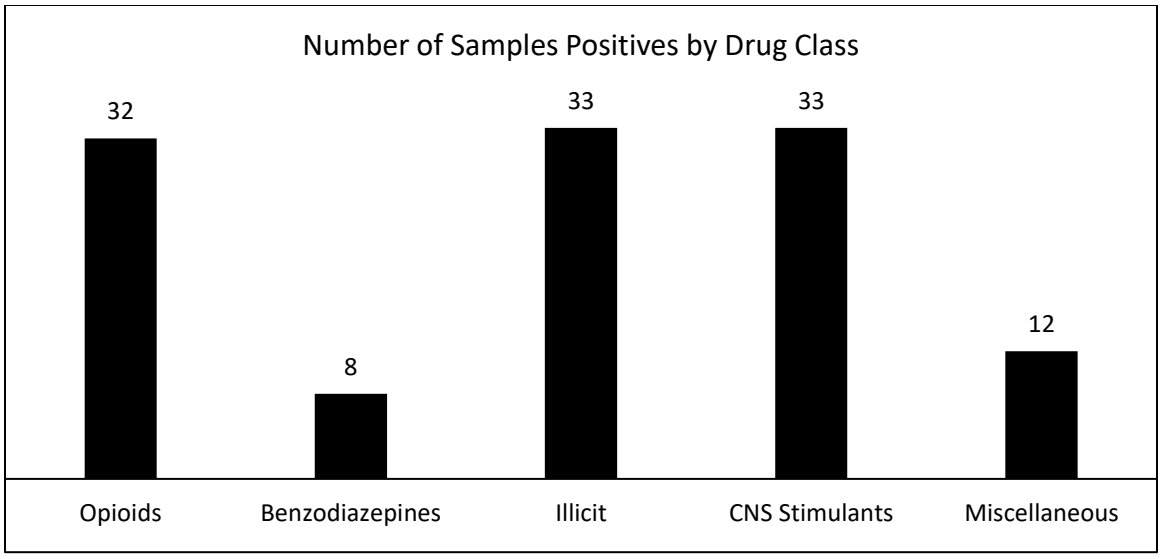

Figure 5. A bar graph representing the number of samples where at least one drug from a specified class was detected.

CHAPTER V

DISCUSSION

5.1 Interpreting Final Results

In order to properly interpret the drug concentrations obtained from this study, the relationship between parent drugs and their metabolites should be understood. The presence of both the parent drug and any screened for metabolites will prove important in correlating WBE data back to the sampled population. Some pharmacologically active metabolites are prescribed on their own and thus observed concentrations may not necessarily be correlated to the parent compound. Other metabolites can be the product of various different parent compounds. While the results of this study provide a proof of concept more than anything else, discussion as to which metabolites could be the result of parental drug use only and which may have been consumed in the metabolized form is important.

5.1.1 Opioids

The consumption of the opiate codeine can sometimes be difficult to differentiate from the use of its major metabolite morphine and minor metabolite hydrocodone[24,30]. Both hydrocodone and morphine are metabolized to some extent into hydromorphone[27]. Hydrocodone use can be differentiated from morphine use by the presence of its unique metabolite norhydrocodone[27].

Codeine was detected in samples 5, 9, 14, 19, 20, 25, and 29 at concentration below 0.5 ng/mL. Samples 5 and 19 contained only the parent compound and no metabolites.

Samples 14 and 20 also contained codeine's major metabolite morphine at a similar concentration, although the concentration of morphine in sample 20, 1.2 ng/mL, was much higher than codeine, 0.1 ng/mL. The metabolite of minor morphine and hydrocodone, hydromorphone, was also detected in sample 20. Both samples also contained codeine's minor metabolite hydrocodone and its subsequent metabolite norhydrocodone at similar levels to both codeine and morphine, 0.1 ng/mL. This indicated the possibility of metabolite use in both samples, along with the consumption of codeine directly. Hydrocodone was also detected in samples 9, 25, and 29 at concentrations similar to codeine, indicating the possibility of its use within these samples as well.

Morphine without the presence of codeine was detected in samples 3, 8, and 23. Hydromorphone was also detected in sample 8. This indicates that the morphine detected in these samples is more likely the result of direct use or as a metabolite of another opioid such as heroin. The metabolite of heroin 6-MAM was not detected in any samples and as such the presence of morphine cannot be related back to heroin. This could be the result of poor stability for 6-MAM within wastewater however. Hydrocodone was detected without the presence of codeine in samples 1, 2, 3, 4, 6, 8, 11, 12, 17, 21, 22, 23, 24, 26, 30, 31, and 32 along with norhydrocodone in samples 1, 2, 6, 8, 21, and 23. This indicates that hydrocodone is likely present in these samples due to its direct consumption than as a product of codeine use within the samples population.

The other opioids screened for within this study are less difficult to differentiate. Oxycodone, a semisynthetic opioid, is metabolized into both noroxycodone and oxymorphone[31]. Buprenorphine, another semisynthetic opioid, has the major urinary metabolite norbuprenorphine. Methadone's major metabolite is EDDP[24]. The first fully synthetic opioid meperidine's metabolite is normeperidine[24]. The major metabolite of the synthetic opioid fentanyl present in urine is norfentanyl[32]. Norcarfentanil is the major metabolite of carfentanil, but is also the

metabolite of remifentanil, a fentalog used medicinally for general anesthesia[33]. Lastly, O-desmethyltramadol is the major metabolite of tramadol.

Oxycodone was detected in samples 1, 2, 3, 5, 6, 7, 9, 12, 13, 14, 15, 17, 18, 20, 21, 23, 24, 29, 30, 32, and 33. Samples 7, 9, and 24 contained only the parent drug, samples 2, 3, 5, 14, 17, and 20 contained both noroxycodone and oxymorphone, and samples 1, 6, 7, 12, 13, 15, 18, 21, 23, 29, 30, 32, and 33 contained only noroxycodone. None of the samples contained only a metabolite of oxycodone without the parent compound. Methadone was only detected in sample 8 while its metabolite EDDP was detected in samples 8, 13, and 26. Tramadol was detected in samples 2, 3, 6, 7, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 27, 29, 30, 32, and 33 while it's metabolite O-desmethyltramadol was only detected in samples 3, 6, 7, 13, 14, 15, 24, 25, 29, and 30. None of the samples where O-desmethyltramadol was detected did not also contain tramadol. The parent compounds buprenorphine, meperidine, fentanyl, and carfentanil and their metabolites were not detected in any of the samples.

5.1.2 Benzodiazepines

The majority of benzodiazepines screened for in this analysis do not share metabolites with the exception of diazepam. Diazepam is metabolized into nordiazepam and temazepam[34]. Temazepam is then further metabolized into oxazepam[35]. Only of these 4 analytes screened for in the analysis, only temazepam was present in any samples. Some other benzodiazepine metabolites included in this screen worth discussing are α-hydroxyalprazolam and 7-aminoclonzepam. Alprazolam is metabolized into α-hydroxyalprazolam but neither were detected in any samples. 7-aminoclonzepam is the metabolite of the benzodiazepine clonazepam, which was not included in this studies analysis screen since it is often difficult to detect in urine[36]. Only sample 1 contained a detectable concentration of 7-aminoclonzepam.

5.1.3 Stimulants

The only non-illicit CNS stimulant metabolite screened for in this study was amphetamine, which was detected at some level in every sample. Amphetamine is also the metabolite of the illicit substance methamphetamine which was not detected in every sample. As such, it's difficult to draw a conclusion as to the cause of the prevalence of amphetamine observed.

5.1.4 Illicits

None of the illicit metabolites screened for can result from numerous parent drugs with the exception of amphetamine which itself is a commonly consumed parent compound. Methamphetamine was present in samples 5, 6, 11, and 14 while amphetamine was present in every sample. Therefore, it can be assumed that at least some of the amphetamine observed in samples 5, 6, 11, and 14 was the result of methamphetamine metabolism. The metabolite of cocaine, benzoylecgonine, was present in every sample while the parent compound was also present in every sample except sample 31.

5.1.5 Background Samples

The background samples were intended to provide a baseline concentration of any drugs or metabolites that might be residually present in the wastewater system prior to the UF football game. Table 18 provides a useful side-by-side comparison between 3 randomly selected game day samples from each sampling location and the background samples corresponding to those locations.

Background 1, 2, and 4 all contained around 0.3 ng/mL of the cocaine metabolite benzoylecgonine, indicating the possibility of a similar baseline concentration. As mentioned in section 5.2.4, benzoylecgonine was present in every sample, however the majority of samples were greater than 0.3 ng/mL. In samples 26, 28, 31, 32, and 33 however, benzoylecgonine was

detected at a concentration below 0.3 ng/mL. The parent compound, cocaine, was also detected at a concentration of 0.1 ng/mL in background 2. Again, nearly every sample contained a detectable concentration of cocaine greater than 0.1 ng/mL with the exception of sample 32. Morphine was detected at a concentration of 0.1 ng/mL in both background 2 and 4. Similar concentrations of morphine were detected in samples 8 and 14. Tramadol was also detected in background 2 and 4 at a concentration of 0.1 ng/mL and 0.2 ng/mL respectively. Sample 17 also contained a concentration of tramadol at 0.2 ng/mL. Pseudoephedrine was detected in background 2, 3, and 4 at concentrations 0.4 ng/mL, 0.2 ng/mL, and 1.2 ng/mL respectively. Pseudoephedrine was detected in all 33 samples with many containing relatively high concentrations. In fact, only sample 28 contained a concentration of pseudoephedrine below 1.2 ng/mL at 0.7 ng/mL. Perhaps most concerning was the detection of trazodone in background 2 and 3 at a concentration of 0.1 ng/mL. This is problematic as every sample containing trazodone was quantified at a low concentration, the highest being 0.3 ng/mL. Samples 3, 11, 15, 20, 22, 23, 24, and 33 all contained a concentration of trazodone near 0.1 ng/mL. Lastly, only background 4 contained a detectable concentration ketamine at 0.1 ng/mL and phentermine at 0.2 ng/mL. Samples 8, 11, 12, 21, 25, and 27 contained a similar concentration of phentermine while the two samples containing ketamine, 8 and 23, were quantified at a slightly higher concentration of 0.3 and 0.2 ng/mL.

In conclusion, the presence of these compound in the background samples indicates the possibility that their detection within the aforementioned samples was the result of a baseline concentration rather than active excretion by the sampled population. However, further background samples will need to be obtained in future studies to better identify these baseline concentrations. Overall, the background concentrations observed when the stadium was not in use are far lower than those observed during the UF vs UK game.

Table 18. Comparison between selected game day samples 7, 8, and 9 and background samples corresponding to their sample collection location.

Compound Name	Sample 7	Background 1	Sample 8	Background 3	Sample 9	Background 4
Benzoylecgonine	2.1	0.2	1.9	-	1.4	0.3
Cocaine	1.5	-	0.4	-	1.6	-
Ketamine	-	-	0.3	-	-	0.1
Morphine	-	-	0.1	-	-	0.1
Phentermine	1.3	-	0.1	-	3.3	0.2
Pseudoephedrine	6.0	-	3.0	0.2	23	1.2
Tramadol	2.1	-	-	-	-	0.2
Trazodone	-	-	-	0.1	-	0.1

5.2 Comparison with Other Sources

One of the major goals of WWBE is to verify drug abuse data already obtained through less reliable sources. One of the best sources of this information comes from SAMHSA, who issues annual nationwide drug use reports broken down from the national level to regional and state levels. Table 18 contains the illicit drug abuse data released in SAMHSA's 2016-2017 annual report for the state of Florida[4]. The most reportedly abused drug is cannabis, which was not analyzed for in this study. However, the next most reportedly misused drug was pain relievers. Many of the opioids detected in the 33 waste water samples analyzed, such as hydrocodone, oxycodone, and tramadol, are commonly prescribed for pain relief. While conclusions cannot be drawn about the nature of opioid consumption among the stadium attendees present during the UF home game, their presence in the waste water does not conflict with the SAMHSA report. Additionally, cocaine was the second most commonly reported illicit drug abused. Cocaine and its metabolite benzoylecgonine were detected in all 33 of the game day waste water samples. This again appears to agree with the SAMHSA report. Lastly, methamphetamine followed by heroin were listed after cocaine as drugs abused by the drug abusers polled by the surveys used in SAMHSA's report. Methamphetamine was detected in 4 samples, alongside its metabolite amphetamine which was detected in every sample. This would again imply that some methamphetamine use is occurring among the attendees excreting drugs

and metabolites into the stadium waste water system. No 6-MAM, the metabolite of heroin, was detected in the analyzed samples but the metabolite of 6-MAM, morphine was present. While the presence of morphine does not necessarily indict heroin use, its presence at least does not rule out the possibility of heroin use and thus does not conflict in any way with the SAMHSA report.

Table 19. Select data obtained from the SAMHSA 2016-2017 annual drug use report for the state of Florida[4].

Measure	Percentage of Users 12 Years of Age or Older
Past Year Cannabis Use	13.69
Past Year Misuse of Pain Relievers	4.18
Past Year Cocaine Use	1.96
Past Year Methamphetamine Use	0.47
Past Year Heroin Use	0.29

5.3 Issues Encountered

5.3.1 Validation of Waters HPLC-MS/MS Method

During the method development stage on the Waters HPLC-MS/MS, validation of calibration linearity was attempted on 2 separate occasions with no success. According to OSU-FTTL validation guidelines, a minimum of 6 calibration curves containing at least 6 concentration levels evenly spaced over the entire quantification range are required for validation. On both occasions multiple analytes contained less than 6 calibration points within the 20% concentration accuracy range while those outside that range were excluded.

There are three possible reasons for these inconsistencies. The first possibility is instrumental error either within the HPLC or MS/MS. After developing a working chromatographic and quantitation method on the Waters, numerous instrumental errors occurred and were addressed. The first major problem presented itself in the form of RT shifts between each analysis run and was diagnosed as a LC pump failure. After replacing the pump hardware

this issue disappeared. Later during the first validation attempt some analytes would be missing from the 400c calibrator while be present in the other calibrators. This issue was partially addressed by updating the instruments software, replacing a faulty guard column, and adjusting the MS/MS tune file. However, similar issue would still occasionally occur during subsequent analyses. Misinjection by the autosampler was ruled out by reinjecting samples and by the fact that only select analytes were not being detected. Developing a method on a different instrument such as the Shimadzu 8040 would help eliminate this as a possible source of error.

The next possibility is SPE error. There were several attempts to troubleshoot the SPE method with some success. Originally the SPE procedure developed for this study contained no pre-conditioning step because the SPE cartridges were said to be designed without a need for preconditioning. After speaking with SPEware technicians the preconditioning step described in Chapter III was introduced. After testing the updated solid phase extraction method, less variability was observed, but it was still present. The second variability in the SPE process was that of flow rate. While uniform pressure was applied to each cartridge during sample addition via the positive pressure manifold, variability in flow rate occurred, even among the calibrators which contained no particulate debris. It's possible that the use of a different SPE cartridge could address this issue.

Finally, a third possible cause of variability in calibrator concentrations post extraction could be human error. There are several opportunities within the extraction and analysis process to introduce human error but the most likely source is during solution preparation and SPE. While this is difficult to control for, an additional extraction on the same system showed that the results were consistent among multiple analysts. Therefore, while this did appear to reduce some of the observed variability, many analytes still required one or more calibrators and QCs to be excluded from both the Waters and Shimadzu analysis.

5.3.2 Cannabinoids

As seen in Tables 8 and 9 of chapter III, the illicit compound THC and its major metabolite THCA were originally included in the LC-MS/MS method developed of this study. However, post extraction concentrations were observed to be very low with only the top three calibrators containing a detectable peak, often not within specified parameters. It's likely that both THC and THCA are either lost at some point during the SPE procedure or retained by the cartridge sorbent during the elution phase. The acidification of the wastewater samples prior to addition and of the sorbent during preconditioning were done partially in an attempt to address this, with little success. Collecting WWBE data on THC and THCA, both compounds found within cannabis, is desirable because of the recent decriminalization of this schedule I substance at the State level. One proposed way to address the inability to extract these analytes using the current SPE method is to develop a separate SPE or liquid-liquid extraction (LLE) method to target both compounds.

5.4 Future Work and Conclusions

Future work on this study will focus on developing a validated LC-MS/MS method, an extraction procedure for cannabinoids of interest, and ways to relate observed concentrations of the analytes of interest back to the sampled population. Additionally, glucuronide metabolites were not included in this studies analysis panel and no glucuronidase enzymes were added prior to SPE of the wastewater samples. A test was performed to see if the SPE procedure would cleave any glucuronide metabolite by extracting and analyzing a water sample containing 3 glucuronide metabolite standards. This glucuronide sample was extracted alongside an extracted positive control, prepared from the calibration stock solution, and negative water control. These three extracted samples were analyzed alongside an unextracted glucuronide water sample on the Shimadzu 8040. The results of this test revealed that the glucuronides had not been hydrolyzed

and were not detectable. Therefore, future studies should also explore the possibility of cleaving any glucuronides likely present in wastewater.

In conclusion, the hypothesis of this study, that drug and metabolite concentrations would be elevated during times when the football stadium is in use when compared to times when it isn't, was confirmed. Overall this work showed that a LC-MS/MS analysis method can be developed for a large panel of drugs of abuse to screen wastewater for the purposes of WWBE. Furthermore, this study also demonstrated that wastewater samples obtained directly from the wastewater system during football games is a promising way to establish drug consumption trends within the attending population. The relative success of this study in identifying 26 of the 56 compounds of interest at a semi-quantitative in at least one of each of the 33 wastewater samples analyzed should lay the foundation for future studies of this kind.

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