THESIS

LC-MS/MS DETERMINATION OF VARIOUS DRUGS OF ABUSE AND METABOLITES IN MUNICIPAL WASTEWATER EFFLUENT SAMPLES

Submitted by

Ryley Adam McPeters

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2012

Master's Committee:

Advisor: Gregory Dooley
Co-Advisor: William Hanneman

Thomas Borch

ABSTRACT

LC-MS/MS DETERMINATION OF VARIOUS DRUGS OF ABUSE AND METABOLITES IN MUNICIPAL WASTEWATER EFFLUENT SAMPLES

A method was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification and confirmation of 8 drugs of abuse (cocaine, codeine, MDMA, methadone, methamphetamine, morphine, nicotine, and oxycodone) and their various metabolites (acetylmorphine, cotinine, EDDP, amphetamine and benzoylecgonine) in municipal wastewater effluent samples. Samples were collected once daily and drugs were extracted from the wastewater with solid phase extraction using Waters Oasis MCX cartridges. Ultra-high-pressure liquid chromatography (UPLC) and positive electrospray ionization was used along with dynamic multiple reaction monitoring (MRM) tandem mass spectrometry for identification and quantitation. The extraction method was validated with matrix matched spiked samples at the limits of quantitation (0.5 ng/mL to 30 ng/mL) for each analyte with recoveries ranging from 70%-140%. Deuterated internal standards for each analyte were used to correct for matrix effects, ion suppression, and sample preparation errors. The validated method was applied to municipal wastewater samples collected from a point source effluent into Fossil Creek, Fort Collins, CO. Eight of thirteen drugs being measured were found on a daily basis with the maximum being 307.72 mg/min of benzoylecgonine. Samples showed various spikes in drug concentration at 7 day intervals that corresponded with weekends.

Key Words: LC-MS/MS, drugs of abuse, metabolites, wastewater

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

Introduction

The use and abuse of illicit drugs has become a problem both nationally and globally in contemporary society. Recent findings from the United Nations Office of drugs and Crime (UNODC) showed that in the "World Drug Report 2011", between 149 and 272 million people between the ages of 15 and 64 consumed an illicit substance at least once in the last year (1). As the abuse of illicit drugs increases, this is turn has incalculable societal consequences which include treatment costs, higher incidence of criminality, and economic damage (2). In recent years, illicit drugs have emerged as a class of environmental contaminants and have begun to catch the attention of certain areas of science including analytical and environmental chemistry, as well as social sciences. Human consumption of illicit drugs is the main way in which these substances contaminate and reach the environment. As these drugs are ingested and metabolized by the body, they are excreted in urine as parent compounds or metabolites. These metabolized drugs then enter wastewater treatment plants (WWTPs) and with the capability to persist in treated wastewaters advance into surface or drinking waters (3).

Drugs of Abuse

A number of illicit drugs and pharmaceuticals are abused on a daily basis and can be seen in trace amounts in wastewater effluent samples (2). The mechanism of action of each illicit drug is unique and well known within the human body. Though little information is known on the ecological effects these drugs and pharmaceuticals have

once they're released into aquatic environments or the potential detrimental effects on organisms that are found in these particular areas. However, both illicit drugs and pharmaceuticals are intended to target similar metabolic and molecular pathways in not only humans but animals as well. When these substances enter into the environment these pathways that are targeted may have effects on animals with similar target organs, tissues, cells, and biomolecules (11).

This study focused on eight commonly abused drugs (cocaine, codeine, MDMA, methadone, methamphetamine, morphine, nicotine, and oxycodone) and their various metabolites (acetylmorphine, cotinine, EDDP, amphetamine, and benzoylecgonine) found in wastewater effluent samples.

Cocaine (benzoylmethylecgonine) is an addictive central nervous stimulant that can be absorbed by the human body either by snorting, injecting, or smoking. When a user takes cocaine they usually experience a euphoric and energetic feeling (4). Once cocaine enters the blood stream it eventually reaches the brain and blocks the reuptake of dopamine, a neurotransmitter associated with pleasure and movement. Cocaine binds to the dopamine receptors blocking reuptake and causing increased amounts of dopamine to flood the brain's synaptic cleft producing large amounts of nerve impulses and an increased sense of exhilaration (5).

Cocaine is one of the most widely abused drugs in the world. Between 14.2 and 20.5 million people abused cocaine between the ages of 15 and 64 during 2009 (1). In humans, urinary excretion makes up almost 90% of cocaine that is ingested with only about 1% of that actually being cocaine that has not been metabolized and remains

unchanged (10). When cocaine is excreted in urine, the majority of it is excreted as its major metabolite benzoylecgonine (13). Benzoylecgonine is produced when cocaine undergoes a cleavage of the methyl ester on the parent compound (10). Approximately 45% of ingested cocaine is excreted as benzoylecgonine(3). Measuring cocaine metabolites in untreated wastewater is also a useful way to identify the route of administration of cocaine, and benzoylecgonine was confirmed to be the best target compound for estimating cocaine analysis in wastewaters because it is the primary human metabolite (12).

3,4-Methylenedioxymethamphetamine (MDMA) is a synthetic, psychoactive drug that has a combination of hallucinogenic and stimulatory properties. MDMA goes by a number of names with the most familiar being ecstasy (6). Ingested orally as a tablet or capsule, MDMA produces effects that include mental stimulation, emotional warmth, enhanced sensory perception, and increased physical energy. MDMA affects the brain by interfering with the neurotransmitter serotonin. Serotonin is responsible for feelings including mood, aggression, sexual activity, sleep, and the perception of pain. Once MDMA is in the brain it binds to the serotonin transporter which cause increased and prolonged amounts of serotonin to enter neuronal synapses. MDMA also has similar effects on other neurotransmitters within the brain including dopamine and norepinephrine which may result in heart rate and blood pressure increases (5).

Methamphetamine (n-methyl-1-phenyl-propan-2-amine) is an addictive stimulant that affects the central nervous system. Similar to cocaine in that methamphetamine affects the neurotransmitter dopamine, this drug has the ability to cause a sense of euphoria along with increased wakefulness and activity, increased heart rate and blood

pressure, and a decreased appetite (6). While cocaine causes the blockage of dopamine reuptake in the synaptic terminals, methamphetamine causes an increased release of dopamine which can be taken up by cells within the brain causing an enhancement in mood and energy. Methamphetamine is a white, odorless powder that can be taken orally, snorted or injected. It can also be used in crystal-like form where it is heated and smoked (5).

Amphetamine (1-phenylpropan-2-amine) is classified as a central nervous system stimulant by itself and has been shown to increase response speed along with retention and recall of verbal memory (9, 10). Amphetamine is most often taken in a tablet or capsule form but can also be crushed up, dissolved in a liquid substance like water and injected intravenously to produce a euphoric feeling along with increases alertness, excitation, and loss of appetite. Because amphetamine is like many stimulants, it works through neurotransmitters, like dopamine, serotonin, and norepinephrine, causing their release while at the same time blocking their reuptake producing a prolonged high (7). Not only is amphetamine abused by itself, it has been shown that when drug abusers use methamphetamine, amphetamine it a metabolic byproduct from liver degradation by cytochrome P-450 enzymes. When methamphetamine is subcutaneously injected there is an increased amount of unchanged methamphetamine and amphetamine excreted in the urine (14).

As pharmaceuticals become more widely available among the general public, the use and abuse of these drugs has become an issue of importance. Morphine [(5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol] is an opioid commonly prescribed as a pain reliever (10). Classified as a Schedule II drug, due to its high

potential for abuse or addiction, morphine has a high physical dependence when used for nonmedical purposes. Morphine can be used a number of ways which include injection, orally as a tablet, or even smoked. People who abuse morphine experience a number of effects ranging from euphoria to respiratory depression and even possible drowsiness (7). Morphine, like other opioids, acts by mimicking the actions of endorphins within the brain and also by binding to opioid receptors found throughout the body. Once morphine reaches these receptors it block the transmission of pain messages to the brain producing a euphoric feeling by affecting areas throughout the brain that mediate the perception of pain (9).

Codeine [(5alpha,6alpha)-7,8-Didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol] is a pharmaceutical that falls under the category of a narcotic or opioid, much like morphine. While it is used as an analgesic like morphine, codeine is not nearly as effective and in most cases used for less severe pain and as a cough suppressant (10). Like other opioids, codeine also works by binding opioid receptors throughout the body and blocking the transmission of pain messages from the body to the brain (8). Codeine is only a partial opioid agonist resulting in a much lower potential for overdose however it does produce opioid-like effects such as euphoria and respiratory depression (7).

Another member of the opioid family is oxycodone (4, 5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one hydrochloride). Oxycodone goes by many other names such as oxycondone, oxymorphone, or oxycontin (8, 10). Similar to morphine, oxycodone is extremely effective in controlling moderate to severe pain and has a long duration of effectiveness (10). Oxycodone is a Schedule II drug only available through

prescription due to its high potential for abuse much like methamphetamine and cocaine (8). Since its introduction in 1995, oxycodone has been linked to multiple overdose fatalities in which abusers crush the pills, and swallow, inhale, or inject the substance resulting in an immediate and intense reaction (7). With the same mechanism of action as other opioids, like morphine and codeine, oxycodone binds to the μ-opioid receptor in the brain causing a euphoric and drowsy feeling. It also binds to the κ-opioid receptor producing adverse effects such as hallucinations while at the same time being able to block the perception of pain throughout the body (9).

Opioids have an extremely high potential for addiction and dependence and this was first recognized during World War II when access to morphine was cut off. A new synthetic narcotic agonist was developed for opioid addiction known as methadone (6-(dimethylamino)-4,4-diphenyl-3-heptanone hydrochloride). Since World War II, methadone has been used in hospital and clinical settings to help systemically detoxify people who are addicted to opiates (7). Although originally used to treat addiction, methadone has emerged as a drug of abuse due to it opioid-like effects. There are a number of ways that methadone can be taken including tablet, oral solution, and even as an injection. Methadone's effects have a slower onset and last much longer than standard opioids which make it a successful way to slowly wean addicts off of other opioids (8). Like other opioids, methadone works through the μ-opioid receptors throughout the body, although a large majority of these receptors are found in the brain, which are responsible for pleasure (4). Methadone is metabolized in the body to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) by cytochrome P-450 cyp3A4

through N-demethylation (13). After the methadone is metabolized to EDDP it is eliminated by the kidneys and is a good urinary biomarker for methadone abuse (15).

One of the most powerfully addictive substances in the world is nicotine. Nicotine's addictive capabilities make cigarette smoke and chewing tobacco the leading cause of preventable death (4). Nicotine (3-(1-Methyl-2-pyrrolidinyl) pyridine) is just one of approximately 4,000 chemicals found in tobacco smoke. It is estimated that about one percent of the weight of a single tobacco leaf is made up of nicotine and if the amount of nicotine in a single cigarette were absorbed quickly into the body, the effects could be extremely toxic and even fatal (7). Nicotine has also been used as an insecticide since 1746. Nicotine is classified as a stimulant because of its abilities to be absorbed through the skin and mucous membranes in humans (7, 10). Like acetylcholinesterase inhibitors, which prevent the cholinesterase enzyme from breaking down acetylcholine, nicotine increases both the level and duration of action of the neurotransmitter acetylcholine within the brain. Nicotine produces symptoms such as vomiting, muscular weakness and possible cardiac fibrillation (10). The mechanism of action for nicotine is complex and begins with nicotine binding to nicotinic cholinergic receptors located on neurons in the brain. This results in the release of the neurotransmitters acetylcholine and glutamate, while at the same time indirectly causing the release of dopamine. Nicotine also has the ability to stimulate the adrenal glands to release epinephrine resulting in an increase in blood pressure, respiration, and heart rate (7).

Like many of the other drugs of abuse, nicotine is also metabolically transformed within the body. The metabolite found in the highest concentration after nicotine use is

cotinine which is the main urinary biomarker for exposure to tobacco smoke (16). Cotinine has the ability to bind to the nicotinic cholinergic receptors found throughout the brain much like nicotine and is highly detectable in blood and urine for multiple days after tobacco use (17). Higher levels of cotinine have been found in the blood from people who come from African descent compared to Caucasians and it has also been shown that people who smoke menthol cigarettes have higher levels of cotinine in their blood as well (18).

One of the most highly abused drugs in the world is heroine. This Schedule I drug is typically taken by intravenous or subcutaneous injection and has both analgesic and euphoric capabilities (7). Heroin has little effect on brain receptors and the body; the pharmacological effects of heroin's active metabolites are what make it extremely addictive. Heroin breaks down into two major metabolites which are morphine and acetylmorphine (15). Heroin is converted to morphine once it crosses the blood-brain barrier. Morphine activates dopaminergic neurons resulting in a sustained activation of the brain's reward pathway leading to a feeling of euphoria (5). Acetylmorphine has a half-life in the body between 6 and 25 minutes and readily enters the brain due to its lipid solubility. Detection of 6-acetylmorphine in the urine is indicative of heroin use and when there are higher concentrations this may indicate chronic use of the drug (15).

Wastewater Treatment Plants

The consumption of illicit drugs and pharmaceuticals is national problem, but not only are these substances harmful to the user; they also have the capability to reach the environment. After a person takes one these substances, a large portion of the drugs

and their metabolites are excreted in the urine. Upon urination, these drugs make their way to wastewater treatment plants and eventually reach the environment through wastewater effluents in measurable quantities.

Wastewater treatment was originally developed to control pollution within the United States (21). Through a combination of physical and biological processes, wastewater treatment was designed to remove organic material from the solutions that are brought into WWTPs. In the U.S., wastewaters are collected from homes, businesses, and industries, and delivered to WWTPs through a large array of collection sewers and pumping stations (19, 21). By providing a buffer between concentrated wastewater and the natural environment in many urban areas, treatment plans release water in a controlled manner. If it weren't for WWTPs, wastewater would degrade water quality, land resources, and the air in which multiple forms of life depend on (22).

The wastewater treatment process is very complex and can be broken down a number of ways, but many ways consist of a very similar process overall. The most processes of a WWTP consist of preliminary, primary, secondary, and finally tertiary treatment. The first step once the water has reached the wastewater treatment facility through the multiple water transfer structures is preliminary treatment. During preliminary treatment, the waste passes through screens or bar racks which help to remove larger debris that may later hinder downstream processes. Some of the larger debris that may be removed consists of wood, cardboard, rags, and other plastic or paper products (22). Next, the water travels to a grit tank where the water flow is slowed down and in some instances chlorine is added to control odor and aid in the settling of solids like sand, rocks, and other solids that passed through the preliminary

screens (20). The solids that have collected within the bar screens and grit tanks are then removed, washed, and taken to the local landfill (19, 23).

After preliminary treatment, the next step in the wastewater treatment process is primary treatment. During this stage, suspended and floating material is removed from the water (22). The water is sent to a sedimentation tank where the water flow is stopped and suspended solids sink to the bottom of the tank and floatable material migrates to the surface of the water. The solids that settle to bottom form a mass known as sludge. Other materials, such as oil and grease, that float to the surface are removed by rotating skimmers in the sedimentation tanks (20, 21). Other biosolids that do not form sludge are removed by pumps and may later be used as fertilizers, removed and sent to landfills, or incinerated (21).

The third step in wastewater treatment is secondary treatment. Secondary treatment helps reduce the concentration of dissolved and colloidal organic substances and suspended matter remaining in the wastewater (22). The majority of secondary treatment involves biological treatment of the wastewater. During this phase, water is mixed with oxygen which starts a process known as aeration which takes place in aeration tanks. Activated sludge, which is bacteria that has become activated due to the presence of oxygen, begins feeding on waste solids and incoming organic matter, thus clarifying the water even further by converting the organic matter into useless by-products (20, 21, 22). When activated sludge isn't used in certain wastewater treatment plants, another approach which consists of trickling filters is employed. Trickling filters usually consist of a bed of stones between three and six feet deep in which the wastewater is passed through. Bacteria grow on these stones and removes organic

matter within the wastewater as it passes through, similar to activated sludge (21). After the trickling filter or activated sludge stage, the water is then sent to a clarifying or settling tank. Here the water is allowed to sit and the excess bacteria and activated sludge microorganisms are removed. When plants use activated sludge, the excess that is removed in the sedimentation tanks is often recirculated back to the aeration tanks to keep the biological process going (20, 21).

The final step in the wastewater treatment process before the water is expelled into the receiving watercourse is tertiary or advanced treatment. During this step, the water that has been expelled from secondary treatment is treated with chlorine or run under high intensity ultraviolet light in order to kill harmful bacteria, viruses, different forms of microorganisms, and amoebic cysts that have been able to make it through the previous treatments (20, 22). When chlorine is used as a disinfectant, the water in many states must also go through a dechlorination phase in order to rid the water of the added chlorine before it is released into the environment (21). Ozonation has also been used in advanced treatment to help remove bacterium and other harmful substances similar to chlorine's effect (23). Tertiary treatment also helps to remove nitrogen and phosphorus within the wastewater before it is expelled because these two elements increase algae growth and may deplete oxygen levels in effluents (20). The use of disinfection techniques throughout the entire wastewater treatment process helps to prevent outbreaks that may be associated with waterborne diseases such as typhoid, cholera, paratyphoid, bacillary dysentery, poliomyelitis, and infectious hepatitis (22).

Once the wastewater has went through the complete cycle of treatment, which may take anywhere between eight and sixteen hours, it is often expelled into a receiving

body of water (20). Wastewater effluents can be used for industrial, agricultural, recreational purposes or even as drinking water sources after another advanced form of water treatment (21). The discharge of effluent wastewater is a significant part of the wastewater treatment program and it can be beneficially used in more than one way such as irrigation or hydroelectric power (22).

The WWTP in Fort Collins, Colorado enforces a similar treatment process to the one described previously that includes preliminary, primary, secondary, and tertiary treatment. During secondary treatment at the Fort Collins plant, the activated sludge that has been used is sent to an anaerobic digester where it is heated to produce methane gas, which is later used for the wastewater treatment process and is also used to heat the facility. This process is not employed by all treatment plants. Biosolids created during the treatment process at this plant, also go through a dewatering process before they are trucked to Meadow Springs Ranch and used as a soil conditioner. After the treatment process, water is expelled into the Fossil Creek Ditch where it is used by farmers for irrigation and it is also used by Rawhide Power Plant as part of an agreement for reuse (24).

Sample Collection

The sampling of wastewater effluents is a relatively new way to analyze illicit drug and pharmaceutical usage. This particular strategy has been incorporated in multiple different countries throughout the world. There are a number of ways to collect samples from wastewater treatment plants and analyze them for their illicit drug content (2).

There are three main ways in which wastewater samples are collected: polar organic chemical integrative sampling (POCIS), composite sampling, and grab sampling. POCIS are normally placed at the area in which the samples are collected from for several days up to a month in wastewater (25). Designed to sample water-soluble organic chemicals from aqueous environments, these are passive devices with no moving parts. POCIS consists of a sorbent found between two polyethersulfone membranes that allow water to pass and capture chemicals of interest (28). These sampling devices have been shown to provide good recovery results when looking at alylphenols amongst other pharmaceuticals (26). POCIS provide a cheaper and efficient way to measure the time weighted average (TWA) of different analyte concentrations. There are two major forms of POCIS, a generic form used mostly for capturing pesticides, as well as other natural and synthetic hormones, and a pharmaceutical form. The analytes captured on the membrane are removed by a solvent extraction, normally methanol depending on the study protocol (27, 28). The disadvantages of these sampling devices are they must remained submerged during the entire sampling time, they must not be buried within the sediment, they are best kept in the shade during sampling periods to prevent chemical degradation, and they are often vandalized (28).

Another method to acquire aqueous samples to monitor illicit drug and pharmaceutical content is through composite sampling. Composite samples are usually taken over a predetermined time period, most often 24 hours. Once the multiple samples have been collected they are pooled together and analyzed, rather than on an individual basis (29). The rise in the use of composite sampling was prompted by

demands in environmental communities to follow multiple federal regulations, and to help clean-up the multiple hazardous waste sites in the country (30). The use of composite sampling has recently been used in studies to measure the use of illicit drugs and pharmaceuticals in wastewaters (3, 13). Composite sampling also helps to significantly reduce the cost of analytics, by reducing the number of samples leading to cheaper environmental and public health assessments (30).

The third technique used for wastewater sampling is by use of the grab sample. Similar to composite sampling in that multiple samples are taken, except during grab samples each individual sample is analyzed by itself. The use of grab sampling assumes that over time there is uniform chemistry within the water and that each sample represents the chemistry at that time and sample position (31). Open discharge pipes where effluents are released into the natural environment are suitable as grab sampling ports (32).

The location where samples are collected plays a major role in the concentration of the particular substances of interest. Samples in wastewater studies are most often either takes as influent wastewater samples, or effluent wastewater and surface water. Influent samples are taken prior wastewater treatment and are used to estimate community consumption. Influent samples are taken prior to wastewater treatment and are used to estimate community consumption. Effluent samples and surface water are taken once the water has been expelled from the wastewater treatment plant and are indicative of wastewater treatment functionality and persistence of substances within the natural environment (2).

Sample Preparation

After samples are collected they must be stored prior to analysis depending on the capture technique. Samples are usually stored in dark areas with temperatures under 4°C, but if samples must be stored for longer periods of time they are usually kept at temperatures around or below -20°C (2). The pH of collected samples is important and samples with a neutral pH are reduced to a pH of 2 upon collection and before they are stored to prevent degradation of cocaine to benzoylecgonine (38, 39, 40).

After samples have been collected, there are two predominant ways to prepare samples prior to analysis, solid phase extraction (SPE) and direct injection. The use of sample preparation is done in order to help adjust for matrix effect that may cause later ionization issues during mass spectrometry analysis. Proper sample preparation can aid in good analyte recoveries and low method limit of detection and quantification (2).

Prior to solid phase extraction a filtering step of the particular wastewater sample in often needed to remove solid particles. Then the pH of the sample is often adjusted, if the sample was not previous adjusted, depending on the SPE protocol (2). Next, the water sample is run through a sorbent cartridge. There are a number of different cartridges that are used however in order to try to isolate varying substances from the wastewater in which they are found. The three most common cartridges used are the Oasis MCX, Oasis HLB, and Strata XC (2).

One of the most widely used cartridges for looking at pharmaceutical and illicit drug content in wastewater samples is the Oasis MCX cartridge which is a mixed mode polymeric-cation exchange sorbent (33). Mixed-mode cartridges allow for both reversed

phase and ion-exchange retention modes for basic compounds (35). This particular column allows for the separation of both basic and neutral compounds from the sorbent during a basic elution (34).

Another widely used cartridge for wastewater analysis of illicit drugs and pharmaceuticals is the Oasis HLB cartridge. This cartridge is a hydrophilic-lipophilic-balanced reversed phase sorbent used for the extraction of acids, bases, and neutrals from a large array of matrices (35).

A cartridge similar to the Oasis MCX is the Strata-XC cartridge (36). Strata XC cartridges contain strong cationic and polar exchange groups. This allows them to bind a large number of drugs since many of them contain positively charged amine groups (37).

Prior to the samples being loaded onto the cartridge, the cartridge must be conditioned. Conditioning helps to wet or activate bonded phases to ensure consistent interaction between the analyte and the sorbent functional groups (45). Depending on the type of cartridge used, this wash usually consists of methanol and water (34, 36). After the conditioning step, samples are loaded onto the cartridge and allowed to pass through either by gravity or with the help of vacuum. Next, the samples are washed with water that has an adjusted pH, acidic or basic depending on the cartridge. Washing the cartridges helps rid them of sample interferences that may have been retained along with compounds of interest (45). Analytes of interest are eluted off the SPE sorbent into a clean vial with a solvent, normally methanol, or an alkaline methanolic solution, or with other solvents such as acetone or ethyl acetate. The

samples are then dried down under a stream of nitrogen and reconstituted in an appropriate solvent for mass spectrometry analysis (2, 33, 34, 36, 41).

An alternative method to SPE is direct injection, which is much faster and cheaper. Through direct injection, you are able to avoid the lengthy process of SPE and simply inject your sample into LC/MS after centrifugation of the sample. The sample size is much larger than SPE, so direct injection uses a 180 mL large-volume injection (LVI) loaded onto a guard column prior to the analytical column. This process also showed acceptable quantification limits similar to SPE (2, 42).

Analytical Techniques

The validation and determination of illicit drug content in municipal wastewater samples has been determined by two separate ways, liquid chromatography combined with mass spectrometry (LC/MS) and gas chromatography (GC) combined with mass spectrometry (GC/MS).

When GC/MS is used to analyze samples of municipal wastewater, there are different steps as opposed to LC/MS. Samples are taken through SPE similar to LC/MS procedures, except when samples are dried down under a stream of nitrogen they are completely dried down and then derivatized with BSTFA ((N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) (43). Derivatization is the process by which a compound is chemically modified to produce a new compound that can be analyzed by gas chromatography. The use of derivatization helps increase volatility, detectability, and improves chromatographic behavior (44). After derivatization, the samples are injected onto a GS/MS instrument with specific protocol parameters (43).

Liquid chromatography combined with mass spectrometry is the most widely used method for the determination of illicit drugs in municipal wastewater samples. Two types of liquid chromatography have been used to evaluate illicit drugs in municipal wastewater samples: high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) (2).

High performance liquid chromatography and ultra performance liquid chromatography are similar when used during the analysis of illicit drug content in wastewater samples (2). HPLC is an extremely powerful tool in analytical chemistry used to separate, identify, and quantitate compounds in a sample that can be dissolved in a liquid. HPLC uses high pressure to push solvents through a packed column. With the use of column particle sizes of 5 µm and pump pressures up to 6000 pounds per square inch (psi), HPLC has been used to separate different constituents of a compound since the 1970's (57, 59).

Ultra performance liquid chromatography is a variant of HPLC. UPLC is a much newer technology that has significant increases in resolution, speed, and sensitivity in liquid chromatography. UPLC uses smaller columns with 1 or 2 millimeter internal diameters packed with smaller particles (1.7 micron) and have the ability to deliver mobile phases at 15,000 (psi) (59). Using high-pressure fluidics and smaller particle size columns, along with the optimization of pump, injector, column, and detector technology, UPLC has improved liquid chromatography (57, 59).

There are three major types of chromatography used within liquid chromatography: hydrophilic interaction liquid chromatogrphay (HILIC), reversed-phase

liquid chromatography (RPLC), and normal phase liquid chromatography (NPLC). Normal phase chromatography is used to separate compounds based on their polarity. NPLC uses a polar stationary phase or column, which is most often silica, in combination with a non-polar solvent. Solvents usually include hexane, ethyl acetate, or other mobile phases that have a low polarity (57). When NPLC is used, non-polar compounds are eluted off at a faster rate than polar compounds (58).

Reversed-phase chromatography involves the separation of molecules based on their hydrophobicity. Columns that are used consist of an alkylsilica-based, non-polar sorbent linked with carbon-18 (C18) that allows separation based on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the sorbent (56). Other columns may be used such as carbon-8 or cyano, both of which have a more immediate polarity. Cyano can be used in both NPLC and RPLC (57). Two separate mobile phases are used for the separation of molecules. One mobile phase consists of a mixture between water and an organic solvent. The other mobile phase is an organic solvent, methanol or acetonitrile, used to elute analytes from chromatographic columns. The aqueous phase usually contains ammonium formate or ammonium acetate, and has been acidified with formic or acetic acids. This aids in the ionization of the compounds in the positive ionization mode. The aqueous phase in the negative ionization mode varies from basic, to neutral, or slightly acidic (2, 3, 40, 41, 47).

Hydrophilic interaction liquid chromatography (HILIC) works like normal phase liquid chromatography (3). The stationary phase in HILIC is often more polar than the mobile phase and the analytes typically elute in an order opposite that of RPLC (57).

The phases used in HILIC consist of a polar stationary phase and a highly organic mobile phase, usually methanol or acetonitrile. Water is used as an eluting solvent and resolves polar analytes better than reversed-phased columns. Under these conditions small polar compounds are retained by the stationary phase (38).

The ionization of illicit drugs and their various metabolites with LC-MS/MS has been carried out with electrospray ionization (ESI). The majority of illicit drugs, their various metabolites, and pharmaceuticals are best ionized in the positive mode. Cannabinoids show good responses in both the positive and negative mode. ESI has one drawback however; it is susceptible to matrix effects of analyte ionization signal (2).

Matrix effects often compromise the analysis of samples by LC-MS/MS. Different approaches have been used to account for matrix effects including: matrix-matched standards calibration, sample dilution, and the use of stable isotopically labeled internal standards (46). Most reported methodologies include isotope-labelled internal standards in order to compensate for losses of desired compounds during SPE and/or matrix effects in wastewater matrices (2).

Mass Spectrometry

There are two major types of mass spectrometry that have been incorporated within liquid chromatography for analysis of wastewater effluent samples: single quadrupole MS (Q) and triple quadrupole MS (QqQ) (55). Single quadrupole mass spectrometry contains a single mass filtering quadrupole. This quadrupole works in a selective mode known as Selected Ion Monitoring (SIM). As a set of voltages are applied to the quadrupole this allows for only one ion of a specific mass-to-charge ratio

(m/z) to pass while other ions with different m/z are filtered out. This allows for the detection of a single analyte as it passes through the quadrupole (54).

Triple quadrupole (QqQ) MS incorporates three different quadrupoles as opposed to a single one (54). QqQ works using a mode known as Multiple Reaction Monitoring (MRM) which allows for more selectivity and noise reduction (54). The first of the three quadrupoles filters out a specific precursor ion based on m/z. The second quadrupole acts as a collision cell to produce a product ion by the collision of the precursor ion with a neutral gas, like nitrogen. This process is known as Collision Induced Dissociation (DIC) producing a product ion that is sent to the third quadrupole. The third quadrupole acts similar to the first where only product ions with a specific m/z are allowed to pass while all others are filtered out (54).

There are multiple advantages to using a triple quadrupole as opposed to a single quadrupole. Triple quadrupoles provide a higher selectivity with less interference resulting in less time consuming method development and faster analysis times. There is also a better signal to noise ratio as compared to the single quadrupole providing lower Limits of Quantitation (LOQ) and better accuracy and reproducibility at lower concentrations (54).

Presence of Illicit Drugs in Wastewater Samples

Sewage epidemiology has proved to be a promising tool to help estimate the use of illicit drugs and pharmaceuticals at both the local and national level. The multiple different approaches that can be incorporated in estimating the use of these drugs show real-time data and in-field information on illicit drug abuse (2, 3).

Profiles of cocaine and its various metabolites in wastewater reveal real human excretion and that wastewater analysis can be used to estimate urinary excretion of cocaine along with its metabolites. Cocaine has reached wastewater by other means besides urinary excretion, including airborne particulate matter along with its presence on money (3). A Spanish study conducted in Almeria, used grab sampling to collect sewage treatment plant effluent samples over the course of two months in order to monitor illicit drug content. Samples were analyzed using LC-MS/MS along with selected reaction monitoring (SRM). Concentraton levels found in effluent samples showed cocaine levels to be 171 ng/L and 1010 ng/L for benzoylecgonine (46). Another study conducted in the province of Castellon, Spain used samples collected over the course of a week in both June and July, showed high concentrations of cocaine and benzoylecgonine. Samples were analyzed by UPLC tandem mass spectrometry showing that benzoylecgonine is the most abundant cocaine metabolite in wastewater samples. Cocaine showed constant concentrations between 0.5 µg/L and 0.8 µg/L throughout analysis times. Higher concentrations of cocaine and benzoylecgonine have also been seen on weekends suggesting a preference for the use of this kind of drug on the weekends and during festivities (41).

A week long study of wastewater influent and effluent samples in Castellon, Spain analyzed by UPLC-MS/MS showed large increases, during a special musical event, of MDMA. Concentrations of MDMA were at 27.5 µg/L on a Sunday, following a music event, while the concentration on the prior Thursday was at 3.26 µg/L suggesting this drug may be used more often during certain occasions (41). A single day 24 hour study conducted in multiple WWTPs throughout the state of Oregon revealed the abuse

of MDMA along with cocaine and methamphetamine. MDMA was seen in approximately half of the 96 treatment plants that collected influent samples. The presence of MDMA was significantly more likely to occur in urban areas opposed to suburban and rural areas (62). The use of POCIS over the course of a year, in a Norwegian study, revealed the abuse MDMA. Using LC-MS/MS for analysis, concentrations of MDMA were low but showed a general increase throughout the course of the year indicating an increase in availability of the drug (60).

Methamphetamine and amphetamine seem to follow similar trends throughout the year in Norway. Amphetamine is the urinary metabolite of methamphetamine. Approximately 4-10% of a methamphetamine dose is excreted in urine as amphetamine (60). Amphetamine levels in wastewater are not only attributed to the use of methamphetamine. Pharmaceuticals such as fenthylline, fenproporex, and selegiline are metabolized to amphetamine and excreted in the urine as well (2). A one week long Spanish study in Castellon using UPLC-MS/MS showed large increases in amphetamine during special events, similar to MDMA (41). Another study, conducted in, Almeria, Spain utilized hourly grab samples taken from a local WWTP influent and put through SPE prior LC-MS/MS analysis showed amphetamine levels to be at 496 ng/L (46).

96 different WWTPs collected composite samples over a 24 hour time period in Oregon. Methamphetamine was present at quantifiable concentrations in raw effluent from every treatment plant (62). Methamphetamine is not only an indicator of the abuse of illicit methamphetamine; it is also a metabolic by-product of the pharmaceuticals selegeline and famprofazone (2). A study conducted in a major metropolitan area in the

United States at a WWTP that served almost 1 million people used both influent and effluent composite wastewater samples that were gathered over the course of a week. The samples were put through SPE prior to LC-MS/MS analysis. Numerous illicit drugs including morphine, cocaine, MDMA and methamphetamine were found in influent samples. Only methamphetamine and MDMA were seen in the effluent samples at concentrations of 86 ng/L and 118 ng/L, respectively. The two of these drugs are slightly more resistant to conventional wastewater treatment (61).

Nicotine and its metabolite cotinine are major contributors to the total amount of drugs in both influent and effluent water samples collected over the course of a week in Almeria, Spain. Both nicotine and cotinine were detected in all influent water samples. Nicotine was not seen in one day's effluent water sample but in every other one. The concentration of nicotine and cotinine were 23.3 µg/L and 27.7 µg/L in influent water samples, respectively. The levels dropped off in effluent samples to 17.3 µg/L for nicotine and 9.5µg/L for cotinine (46). A specific study conducted in Zurich, Switzerland looked only at the concentration of nicotine and its metabolites, including cotinine, in influent, effluent, and surface water samples. The samples were put through SPE and analyzed by LC-MS/MS. Cotinine was found in all the samples including surface water samples. The typical amount of cotinine in a smoker's urine is approximately 1.6 mg/L and assuming mean urine production is about 1.5 L/d, this shows that smokers release 2.4 mg/person/day of cotinine into WWTPs (63).

Methadone and its metabolite EDDP can be described as tranquilizing drugs that have also been found at low concentrations in both influent and effluent wastewater samples in Spain (46). In a separate study, grab samples of raw and treated

wastewater were collected and put through SPE. The samples were then analyzed using LC-MS/MS and concentrations of various drugs of abuse were calculated. Methadone and EDDP were seen in both raw and treated wastewater. The concentrations of these two compounds remained relatively consistent throughout the sampling timeframe. This can be attributed to the fact the methadone is used as a medical substitute for heroin addiction and is metabolized to EDDP in the body before they are both excreted in urine (34).

Certain metabolites of drugs may actually be consumed directly or are actually metabolites of other compounds. Morphine, is the major metabolite of heroin, but is also administered directly for the mitigation of pain in medical facilities (61). The metabolite of morphine is acetylmorphine and is often seen in wastewater samples when morphine is present. Three separate studies, all of which were conducted in Spain detected the presence of morphine and acetylmorphine. Each of these studies collected wastewater samples for over a week and the samples were from both influents and effluents. Each sample from the three separate WWTPs was analyzed using LC-MS/MS. Morphine was seen in all influent samples at varying quantities in each study, and was also present in some effluent samples. Acetylmorphine was seen in both influent and effluent samples. The concentration of acetylmorphine was less than that of morphine in all the studies that showed its presence (34, 46, 52).

The presence of codeine was found in wastewater samples collected from a WWTP in Almeria, Spain. Samples were analyzed by LC-MS/MS, and SRM was used for identification and detection of codeine along with other drugs. Codeine was detected at consistent concentrations over the course of a week (700-930ng/L) (46).

Facilities that manufacture pharmaceuticals are often scrutinized for the release of many pharmaceuticals into the environment via wastewater. Oxycodone has been found in wastewater samples found throughout the U.S. In a study conducted in New York, three WWTPs collected grab samples from wastewater effluents. Two of the three treatment plants received flow from hospitals. Samples were subjected to SPE and then analyzed by GC-MS. The presence of oxycodone was seen in each of the three WWTPs where samples were collected. The maximum concentration of oxycodone seen was 1700 µg/L. The two WWTP that had hospitals contributing to the plant had higher concentrations of pharmaceuticals than the one plant that did not have a hospital contributing to the wastewater. Hospitals may contribute a large amount of pharmaceuticals found in wastewater but are not the only contributor (64).

Constant consumptions of drugs including: caffeine, nicotine, cocaine, ephedrine, codeine, morphine, and methadone were seen in both wastewater and surface water samples, revealing the prevalence of these substances in the aquatic environment (46). Concentrations have been found not only downstream but also upstream from WWTPs indicating that wastewater is a significant contributor to pharmaceutical loading in various receiving waters (53).

Illicit drugs are common contaminants of aquatic environments in populated areas. Contamination by illicit drug residues appears to be widespread, and the major source can be attributed to consumers. Although many of these concentrations are low, the presence of drugs and pharmaceuticals in surface waters may lead to pharmacological interactions causing toxic effects to aquatic organisms (13). Particular knowledge on different aspects within the wastewater treatment process including time

of residence of each drug, the amount of light exposure each drug sees, and the temperature conditions would help calculate drug consumption and usage (2).

References:

(1) UNODC. United Nations Office of Drugs and Crime World Drug Report 2011. 2011; United Nations: New York.

(2) Nuijs, A.L.N., Castiglioni, S., Tarcomnicu, I., Postigo, C., de Alda, M.L., Neels, H., Zuccato, E., Barcelo, D., and Covaci, A. 2011. Illicit drug consumption estimations derived from wastewater analysis: A critical review. *Science of the Total Environment*; 409: 3564-77.

(3) Castiglioni, S., Bagnati, R., Melis, M., Panawennage, D., Chiarelli, P., Fanelli, R., and Zuccato, E. 2011. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. *Water Research*; 45: 5141-50.

(4) (NIDA) National Institute on Drug Abuse. August 2010. http://www.drugabuse.gov/

(5) Lunbeck Institute. 2011. *Educational Resources.* *http://www.cnsforum.com/educationalresources/imagebank/*

(6) (DEA) Drug Enforcement Administration. Drug and Chemical Evaluation Section. August 2011. http://www.deadiversion.usdoj.gov/index.html

(7) Abadinsky, H. 2004. Drugs An Introduction. *Thomson and Wadsworth;* 5: 62-166.

(8) Ginther, C. 2004. Drug Abuse Sourcebook. *Omnigraphics.* 2nd Edition.

(9) Nutt, D., Robbins, T.W., Stimson, G.V., Ince, M., and Jackson, A. 2007. Drugs and the Future: Brain Science, Addiction, and Society. *Academic Press.*

(10) Perrine, D.M. 1996. The Chemistry of Mind-Altering Drugs. *American Chemical Society.*

(11) Fent, K., Weston, A.A., and Caminada, D. 2006. Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology;* 76:122-59.

(12) Van Juijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P. G., Bervoets, L., Blust, R., Meulemans, H., Neels, H., and Covaci, A. 2009. Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium. *Addiction;* 104: 734-41.

(13) Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., and Fanelli, R. 2008. Illicit drugs, a novel group of environmental contaminants. *Water Research;* 42: 961-68.

(14) Yamada, H., Oguri, K., and Yoshimura, H. 1986. Effects of several factors on urinary excretion of methamphetamine and its metabolites in rats. *Enobiotica*; 16: 137-41.

(15) Couper, F. J., and Logan, B.K. 2004. Drug and human performance fact sheets. *(NHTSA) National Highway Traffic Safety Administration.*

(16) Bao, Z., Xiao-Yang H., Ding, X., Prabhu, S., and Hong, J. 2005. Metabolism of nicotine and cotinine by human cytochrome P450 2A13. *Drug Metabolism and Disposition;* 33: 258-61.

(17) Dwoskin, L.P., Teng, L., Buxton, S.T., and Crooks, P.A. 1999. (S)-(2)-Cotinine, the major brain metabolite of nicotine, stimulates nicotinic receptors to evoke [H_3] dopamine release from rat striatal slices in a calcium-dependent manner. *The Journal of Pharmacology and Experimental Therapeutics;* 208: 905-11.

(18) Mustonen, T.K., Spencer, S.M., Hoskinson, R.A., Sachs, D.P., and Garvery, A.J. 2005. The influence of gender, race, and menthol content on tobacco exposure measures. *Nicotine Tobacco Research;* 7: 581-90.

(19) Hammer, M.J., and Hammer Jr., M.J. 1996. Water and wastewater technology. *Prentice Hall;* 3rd Edition.

(20) Carlsen, S. 1997. How a house works: What happens after the flush. *The Family Handyman;* March: 107-13.

(21) Environmental Protection Agency (EPA). 1998. How wastewater treatment works… The basics. *Office of Water*.

(22) Management and Support Systems. 1996. Operation of municipal wastewater treatment plants. *Water Environment Federation;* 5th Edition.

(23) Lacrosse, G.R. 1992. UV-Enhanced ozone wastewater treatment system. *United States Patent.*

(24) City of Fort Collins. Retrieved April 2012. Treating wastewater. http://www.fcgov.com/utilities/what-we-do/wastewater/treating-wastewater

(25) van Nuijs, A.L.N., Castiglioni, S., Tarcomnicu, I., Postigo, C., de Alda, M.L., Neels, H., Zuccato, E., Barcelo, D., and Covaci, A. 2011. Illicit drug consumption estimations derived from wastewater analysis: A critical review. *Science of the Total Environment;*409: 3564-77.

(26) Miege, C., Budzinski, H., Jacquet, R.,, Soulier, C., Pelte, T., and Coquery, M. 2011. Polar organic chemical integrative sampler (POCIS): application for monitoring organic micropollutants in wastewater effluent and surface water. *J. Environ. Monit.;* 14: 626-35.

(27) Cernoch, I., Franek, M., Diblikova, I., Hilscherova, K., Randak, T., Ocelka, T., and Blaha, L. 2011. POCIS sampling in combination with ELISA: Screening of sulfonamide residues in surface and wastewaters. *J. Environ. Monit.;* 14: 250-7.

(28) United States Geological Survey (USGS). 2004. Polar Organic Chemical Integrative Sampler (POCIS). *Columbia Environmental Research Center.* http://www.cerc.usgs.gov/pubs/center/pdfdocs/pocis.pdf

(29) El-Shaarawi, A. H., and Piegorsch, W. W. 2002. Composite sampling. *Encyclopedia of Environmetrics;* 1: 387-91.

(30) Lancaster, V.A., and Keller-McNulty, S. 1998. A review of composite sampling methods. *J. of the American Statistical Association;* 93: 1216-30.

(31) Technical Advisory Committee (TCA). 2008. Onsite wastewater treatment sampling: Grab vs. Composite sampling. http://www.mowra.org/TACWastewaterSamplingAdvisory.pdf

(32) American Public Health Association, American Water Works Association, Water Environment Federation. 1998. *Standard Methods for the Examination of Water and Wastewater;* 20th Ed.

(33) Gracia-Lor, E., Sancho, J.V., and Hernandez, F. 2010. Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry. *Journal of Chromotography A;* 1217: 622-32.

(34) Gonzalex-Marino, I., Quintana, J. B., Rodrigues, I., Gonzalez-Diez, M., and Cela, R. 2011. Screening and selective quantification of illicit drugs in wastewater by mixed-mode solid-phase extraction and quadrupole-time-of-flight liquid chromatography-mass spectrometry. *Analytical Chemistry;* 84: 1708-17.

(35) Waters. 2011. Oasis sample extraction products. http://www.waters.com/waters/nav.htm?cid=513209

(36) Bisceglia, K. J., Robers, A. L., Schantz, M. M., and Lippa, K. A. 2010. Quantification of drugs of abuse in municipal wastewater via SPE and direct injection liquid chromatography mass spectrometry. *Analytical and Bioanalytical Chemistry;* 398: 2701-12.

(37) Huq, S., Dixon, A., Teuscher, J., Lok, S., and Kallury, K. 2003. Efficient extraction of basic drugs from biological matrices using a polymeric cationic mixed-mode sorbent – strata X-C. *Phenomenex Inc.*

(38) Gheorghe, A., van Juijs, A., Pecceu, B., Bervoets, L., Jorens, P.G., Blust, R., et al. 2008. Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry. *Anal. Bioanal. Chem.;* 391: 1309-19.

(39) Kaspryz-Horden, B., Dinsdale, R.M., Guwy, A.J. 2009. Illicit drugs and pharmaceuticals in the environment – forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities. *Environ. Pollut.;* 157: 1773-7.

(40) van Juijs A.L.N., Tarcomnicu, I., Jorens, P.G., Bervoets, L., Blust, R., Neels, H., et al. 2009. Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Annal. Bioanal. Chem.;* 395: 819-28.

(41) Bijlsma, L., Sancho, J. V., Pitarch, E., Ibanez, M., and Hernandez, F. 2009. Simultaneous ultra high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. *J. of Chromatography A;* 1216: 3078-89.

(42) Chiaia, A. C., Banta-Green, C., Field, J. 2008. Eliminating solid phase extraction with large-volume injection LC/MS/MS; Analysis of illicit and legal drugs and human urine indicators in U.S. wastewaters. *Environ. Sci. Technol.;* 42: 8841-8.

(43) Mari, F., Politi, L., Biggeri, A., Accetta, G., Trignano, C., Di Padua, M., et al. 2009. Cocaine and heroin in waste water plants: A 1-year study in the city of Florence, Italy. *Forensic Sci., Int.;* 198: 88-92.

(44) Regis Technologies Inc. 2000. GC derivatization. http://www.registech.com/Library/gcderrev.pdf

(45) Sigma-Aldrich. 2011. Reversed-phased methodology. http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/reversedphase-methodology.html

(46) Martinez Bueno, M. J., Ucles, S., Hernando, M.D., and Fernandez-Alba, A. R. 2011. Development of a solvent-free method for the simultaneous identification/quantification of drugs of abuse and their metabolites in environemental water by LC-MS/MS. *Talanta;* 85: 157-66.

(47) Boleda, M. R., Galceran, M. T., and Ventura, F. 2007. Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatagraphy A;* 1175: 38-48.

(48) Basheer, C., Chong, H. G., Hii, T. M., and Lee, H. K. 2007. Application of porous membrane-protected micro-solid-phase extraction combine with HPLC for the analysis of acidic drugs in wastewater. *Anal. Chem.;* 79: 6845-50.

(49) Lurie, I. S. 2005. High-performance liquid chromatography of seized drugs at elevated pressure with 1.7µm hybrid C18 stationary phase columns. *Journal of Chromatography A;* 1100: 168-175.

(50) Farre, M., Ferrer, I., Binebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barcelo, D. 2001. Determinatino of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: Methods and preliminary results including toxicity studies with *Vibrio fischeri. Journal of Chromatography A;* 938: 187-97.

(51) Pozo, O. J., Sancho, J. V., Ibanez, M., and Hernandez, F. 2006. Confirmation of organicmicropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: Achievements and pitfalls. *TrAC Trends in Analytical Chemistry;* 25: 1030-42.

(52) Pedrouzo, M., Borrull, F., Pocurull, E., Marce, R. M. 2011. Drugs of abuse and their metabolites in waste and surface waters by liquid chromatography-tandem mass spectrometry. *J. Sep. Sci.;* 34: 1091-1101.

(53) Bartelt-Hunt, S. L., Snow, D. D., Damon, T., Shockley, J., and Hoagland, K. 2009. The occurrence of illicit and therapeutic pharmaceuticals in wastewater

effluent and surface waters in Nebraska. *Environmental Pollution;* 157: 786-91.

(54) Schreiber, A. 2010. Advantages of using triple quadrupole over single quadrupole mass spectrometry to quantify and identify the presence of pesticides in water and soil samples. *AB SCIEX:* 1-6.

(55) Ferrer, I., and Thurman, E. M. 2003. Liquid chromatography/mass spectrometry, MS/MS and time-of-flight MS: Analysis of emerging contaminates. *Oxford University Press.*

(56) Walker, J. M., and Rapley, R. 2008. Molecular Biomethods Handbook. *Humana Press.*

(57) Wang, P. G., and He, W. 2011. Hydrophilic Interaction Liquid Chromatography (HILIC) and Advanced Applications. *CRC Press.*

(58) Snyder, L. R., Glajch, J. L., and Kirkland, J. J. 1988. Practical HPLC Method Development. *Wiley-Interscience.*

(59) Waters. 2012. HPLC - High Performance Liquid Chromatography. http://www.waters.com/waters/nav.htm?cid=10048919

(60) Harman, C., Reid, M., and Thomas, K. V. 2011. In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage. *Environ. Sci. Technol.;* 45: 5676-82.

(61) Gerrity, D., Trenholm, R. A., and Snyder, S. A. 2011. Temporal variability of pharmaceuticals and illicit drugs in wastewater and the effects of a major sporting event. *Water Research;* 45: 5399-411.

(62) Banta-Green, C. J., Fields, J. A., Chiaia, A. C., Sudakin, D. L., Powers, L., and de Montigny, L. 2009. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater. *Addiction;* 104: 1874-80.

(63) Buerge, I. J., Kahle, M., Buser, H., Muller, M. D., and Poiger, T. 2008. Nicotine derivatives in wastewater and surface waters: application as chemical markers for domestic wastewater. *Environ. Sci. Technol.;* 42: 6354-60.

(64) Phillips, P. J., Smith, S. G., Kolpin, D. W., Zaugg, S. D., Buxton, H. T., Furlong, E. T., Esposito, K., and Stinson, B. 2010. Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. *Environ. Sci. Technol.;* 44: 4910-16.

CHAPTER 2

LC-MS/MS DETERMINATION OF VARIOUS DRUGS OF ABUSE AND METABOLITES IN MUNICIPAL WASTEWATER EFFLUENT SAMPLES

1. Introduction

The use and abuse of illicit drugs has become a problem both nationally and globally in society. Recent findings from the United Nations Office of drugs and Crime (UNODC) showed that in the "World Drug Report 2011", between 149 and 272 million people between the ages of 15 and 64 consumed an illicit substance at least once in the last year (1, 2, 3). As the abuse of illicit drugs increases, societal changes occur which include increased treatment costs, higher incidence of criminality, and economic damage (4). In recent years, illicit drugs have emerged as a class of environmental contaminants and have begun to catch the attention of certain areas of science including analytical and environmental chemistry. Human consumption of illicit drugs is the main way in which these substances contaminate and reach the environment (5). As these drugs are ingested and metabolized by the body, they are excreted in urine as parent compounds or metabolites (6). These metabolized drugs then enter WWTPs and with the capability to persist in treated wastewaters and advance into surface or drinking waters (5). Drugs can also reach wastewater treatment plants (WWTPs) through the disposal of unused or expired products, and from pharmaceutical discharges (6).

A number of illicit drugs and pharmaceuticals are abused on a daily basis and can be seen in trace amounts in wastewater effluent samples (4). The analysis of illicit

drugs in effluents coming from WWTPs has been used to estimate community-level consumption of illicit drugs and abused pharmaceuticals (7). Wastewater treatment plants can not completely remove every contaminant that enters the system (6). Studying drug levels in wastewaters helps proved realistic estimations of drug consumption in various communities in real time. This will help educate the general public and policy makers at the same time to help start prevention campaigns against drugs or other targeted actions (2, 8). Currently, the prevalence and occurrence of illicit drugs is obtained by surveys integrated with crime statistics, medical records, and drug production and seizure rates (4). The use of surveys has many limitations including limited population coverage, self-report bias, and substantial time lags resulting negatively the reliability, validity, and usefulness this data (9). By studying the levels of drugs and their metabolites in wastewater, this helps provide realistic and comparable estimates of drug consumption in various communities (8).

While the mechanism of action of each illicit drug is unique and well known within the human body, little information is known on the ecological effects these drugs and pharmaceuticals have once they're released into an aquatic environment. There are many documented adverse effects at low levels that include acute and chronic damage, accumulation in tissues, reproductive damage, inhibition of cell proliferation, and behavioral changes (10, 11). With incomplete removal of illicit drugs and metabolites at WWTPs, low concentrations of these drugs contaminate surface and drinking water (3). Illicit drugs and pharmaceuticals target similar metabolic and molecular pathways in not only humans but aquatic organisms as well. When these substances enter into the

environment these pathways that are targeted may affect organisms with similar target organs, tissues, cells, and biomolecules (10).

The use of solid-phase extraction (SPE) is the most common method for determining drugs of abuse found in wastewaters. Sorbents, like the Oasis MCX, provide a dual cationic-exchange/reversed phase character aiding in the selectivity of SPE (8). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been proven to be the technique preferred to analyze drugs in wastewater samples due to the polarity of the drugs (8). Recently though, ultra high-pressure liquid chromatography (UHPLC) coupled with MS/MS has become the most suitable analytical tool for determining the presence of contaminants in wastewater samples by providing greater resolution, increased sensitivity, and faster analysis times with MS/MS minimizing or eliminating matrix interferences. The presence of matrix effects in wastewater samples is a main factor affecting the sensitivity of many analytical methodologies (6, 14). The use of isotope-labeled internal standards has been implemented into multiple procedures to correct for matrix effects within samples (6). Deuterated internal standards also provide increased limits of detection (LODs) and quantification (LOQs) (3).

The purpose of this research was to identify drugs of abuse and various metabolites present in Fort Collins, CO municipal wastewater effluent samples that were released into Fossil Creek and the environment. The method that was developed, used Oasis MCX (mixed mode) cartridges for clean-up and pre-concentration followed by UHPLC-MS/MS measurement. This method was applied to daily grab samples collected over the course of 6 months from July to December, a week in early February

which coincided with the Super Bowl, and a 24 hour study conducted in May. Emphasis was put on the confirmation of drugs of abuse within the effluent samples as well as the quantification and confirmation of positive samples.

2. Experimental

Chemicals and Materials

Cocaine, codeine, MDMA, methadone, methamphetamine, morphine, nicotine, and oxycodone and their various metabolites: acetylmorphine, cotinine, EDDP, amphetamine, and benzoylecgonine found in wastewater effluent samples were obtained from Cerilliant (Round Rock, TX, USA) and Agilent Technologies (Santa Clara, CA, USA) as solutions in methanol and acetonitrile. Standard stock solutions for each compound were in 100 µg/mL or 1 mg/mL concentrations. Stock solutions were combined into a mixed working solution at 50ng/mL and 500ng/mL in methanol and were used for preparation of wastewater calibration standards, and for spiking samples in the study used for validation.

Deuterated compounds (cocaine-D3, codeine-D6, MDMA-D5, methadone-D9, methamphetamine-D5, morphine-D6, nicotine-D4, oxycodone-D6, acetylmorphine-D6, cotinine-D3, EDDP-D3 perchlorate, amphetamine-D6, and benzoylecgonine-D3) were obtained from Cerilliant and Sigma-Aldrich (St. Louis, MO, USA) as solutions in methanol and acetonitrile at a concentration of 100 µg/mL. Solutions were combined into a mixed internal standard working solution at 5 µg/mL in methanol.

All standard solutions were stored in amber glass vials at -20ºC. LC-MS grade water, acetonitrile and ammonium formate were obtained from Sigma-Aldrich. LC-MS grade formic acid was obtained from Thermo Fisher Scientific (Waltham, MA, USA). SPE cartridges, mixed reversed-phase/cation-exchange cartridges (Oasis-MCX; 3 mL, 60 mg) were purchased from Waters (Milford, MA, USA)

Sample Collection

Daily grab samples were taken over the course of six months between July and December of 2011 from an effluent from a WWTP in Fort Collins, CO. Samples were taken between the hours of 8 A.M. and 10 A.M. Another week of daily samples was collected during early February 2012 during the week of the Super Bowl. A 24-hour study during May 2012 used grab samples that were collected every 2 hours over the course of a day during the middle of the week. Each sample was collected in a 250 mL glass bottle and acidified to a pH=2 using 37% hydrochloric acid and stored in the dark at 4°C until analysis.

Solid-Phase Extraction

Oasis MCX 3cc (60 mg) cartridges were preconditioned with 3 mL of MeOH, 2 mL of milli-Q water (18 Ω), and 2 mL of acidified water (pH=2). Prior to samples running through the cartridges, 10 µL of deuterated internal standard (5µg/mL) was added to each 170 mL sample. Samples were pulled through under vacuum at 1 drop per second. After the samples had passed through the cartridges, they were washed with 2 mL of 2% NH_4OH and dried for 15 minutes under vacuum. The cartridges were then eluted with 4 mL of 2% NH_4OH in MeOH and evaporated under a stream of nitrogen

until approximately 50 µL remained. The eluates were reconstituted in mobile phase A (5mM ammonium formate/0.01% formic acid in water) up to 200 µL, placed in LC amber glass vials for LC-MS/MS analysis.

Method Validation Before Sample Collection

The acquisition of drugs and metabolites was performed using the MRM mode, with a precursor ion for each compound being used for identification. Two additional product ions were also used for confirmation. Each compound was quantified using a corresponding deuterium labeled analyte as an internal standard. Method accuracy and precision was evaluated by analyzing standard solutions in wastewater in triplicate at six different concentrations (0.5ng/mL, 1 ng/mL, 5 ng/mL, 10ng/mL, 20 ng/mL, and 30 ng/mL). Recoveries between 70% and 140% were considered satisfactory to act as the limit of quantification (LOQ) was determined as the lowest concentration with acceptable precision, accuracy, and recoveries.

LC-MS/MS Analysis

This method looking for 13 illicit drugs of abuse and their various metabolites utilized UPLC-MS/MS with electrospray ionization in the positive mode. The instrument used in the analysis was an Agilent 1290 UPLC coupled to an Agilent 6460 triple quadruple mass spectrometer, which was equipped with an ESI source using Agilent Jet Stream Technology (Agilent, Santa Clara, CA). Drugs and metabolites were separated on a Zorbax Eclipse Plus C18 column (2.1mm x 150mm, 1.8 µm particle size) at 60 °C. A sample volume of 15 µL was injected and a binary mixture of 5mM ammonium formate/0.01% formic acid in water (A) and acetonitrile with 0.01% formic

acid (B) at a flow rate of 0.5 mL/min. The gradient used was 10% B increasing to 95% B at 4 min, and held for 2 min. The ionization source conditions used were as follows: nebulizer gas flow of 8 L/min at 320 °C and 27 psi; sheath gas flow of 12 L/min at 380 °C; and the capillary voltage 3750 V. The optimized fragmentor, collision energy, and MS-MS transitions for each analyte were obtained using the Agilent Mass Hunter Optimizer Software B.04.01. The dwell times for the transitions were maximized based on the number of concurrent MRMs A test mix containing 13 drugs of abuse and their metabolites was run prior to each sample set to calibrate retention time windows. The data collection and processing were performed by using Agilent MassHunter Quantitative software (v B.04.01) (Table 1).

Table 1: UHPLC-MS/MS parameters established for the MRM acquisition mode for the determination of drugs of abuse, various metabolites, and their deuterated internal standards.

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Ret Time (min)	Polarity
Acetylmorphine	328.2	211.1 165	158	21 33	2.5	Positive
Acetylmorphine-D6	334.2	165	151	37	2.5	Positive
Ampthetamine	136.1	119.1 91	66	5 17	2.28	Positive
Amphetamine-D6	142.1	93.1	66	13	2.28	Positive
Benzoylecgonine	290.3	168.3 105.3	70	15	2.65 2.65	Positive
Benzoylecgonine-D3	293.2	171.1	70	14	2.65	Positive
Cocaine	304.2	182.1 77	138	17 61	3.34	Positive
Cocaine-D3	307.2	185.1	138	17	3.34	Positive
Codeine	300.2	165.1 58.1	158	45 29	2.25	Positive
Codeine-D6	306.2	165.1	158	45	2.25	Positive
Cotinine	177.1	98.1 80.1	40	25 25	2.5	Positive
Cotinine-D3	180.1	80.1	40	25	2.5	Positive
EDDP	279.2	250.2 235.1	151	17 29	4.4	Positive
EDDP-D3	282.2	235.1	151	29	4.4	Positive
MDMA	194.1	163 105	97	9 25	2.6	Positive
MDMA-D5	199.1	165.1	97	9	2.6	Positive
Methadone	310.2	265.1 105	112	9 29	4.52	Positive
Methadone-D9	319.3	268.2	112	9	4.52	Positive

Methamphetamine	150.1	119 91	92	5 17	2.44	Positive
Methamphetamine-D5	155.2	92.1	126	17	2.44	Positive
Morphine	286.2	165.1 157.1	126	41 41	1.75	Positive
Morphine-D6	292.2	181	141	37	1.75	Positive
Nicotine	163.1	130 84	120	15 20	1.5	Positive
Nicotine-D4	167.1	134	120	15	1.5	Positive
Oxycodone	316.2	298.1 256.1	143	17 25	2.43	Positive
Oxycodone-D6	322.2	304.2	143	17	2.43	Positive

3. Results

Method Validation

Quantification limits were obtained as the lowest concentration with precision, accuracy, and recoveries. The limits of quantification (LOQ) ranged from 5.9 ng/L to 571.4 ng/L in wastewater samples spiked with standard stock solutions. The difference in recoveries amongst the various analytes may be related to matrix effects and the polarity of each of the analytes. Oasis MCX cartridges were selected for SPE of the wastewater effluent samples due to their mixed mode material capabilities that allows for improved selectivity towards basic compounds due to pH and the changes in polarity during loading, washing, and elution. Deuterated internal standards were added as surrogates in all cases to help reduce matrix effects and compensate for losses during sample preparation. Percent recovery of each analyte along with the coefficients of variation (CV), limit of quantification (LOQ), and the deuterated internal standard used for each analyte can be seen in Table 2. Recoveries ranged from 39 to 141 % and the LOQ were between 5.9 to 571.4 ng/L.

Table 2: Method of validation results for spiked wastewater with recovery (%), coefficient of variation (CV), and Limit of Quantification (LOQ).
(-): indicates the concentration was below the limit of detection (LOD)

Compound	5.9 ng/L		29.4 ng/L		58.8 ng/L		117.6 ng/L		LOQ (ng/L)	Internal Standard used
	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV		
Nicotine	-	-	-	-	105	36.91	72	20.32	58.8	Nicotine-D4
Morphine	-	-	90	9.14	107	15.11	92	4.39	29.4	Morphine-D6
Codeine	-	-	77	9.7	99	15.17	83	2.26	29.4	Codeine-D6
Benzoylecgonine	-	-	70	0.73	79	2.38	98	5.72	29.4	Benzyolecgonine-D3
Cotinine	97	9.81	81	12.54	96	15.01	82	2.69	5.9	Cotinine-D3
Amphetamine	91	14.71	82	11.29	92	15.27	78	1.73	5.9	Amphetamine-D6
Oxycodone	-	-	98	9.07	141	26.48	93	2.52	29.4	Oxycodone-D6
Methamphetamine	-	-	39	12.02	48	18.09	45	2.44	571.4	Methamphetamine-D5
MDMA	104	16.58	91	14.79	93	15.56	89	1.63	5.9	MDMA-D5
Acetylmorphine	130	16.05	110	11.18	123	14.55	107	2.81	5.9	Acetylmorphine-D6
Cocaine	93	14.38	86	11.46	98	17.3	87	1.49	5.9	Cocaine-D3
EDDP	-	-	92	6.39	112	19.2	87	3.84	29.4	EDDP-D3
Methadone	123	15.23	88	8.57	97	18.56	84	2.41	5.9	Methadone-D9

Illustrative chromatograms are shown in Figures 1 and 2 for a wastewater sample spiked with 100ng/mL stock solution and a sample collected on July 30th, 2011. The sensitivity of this method and its potential to detect low levels of analytes proved to be excellent.

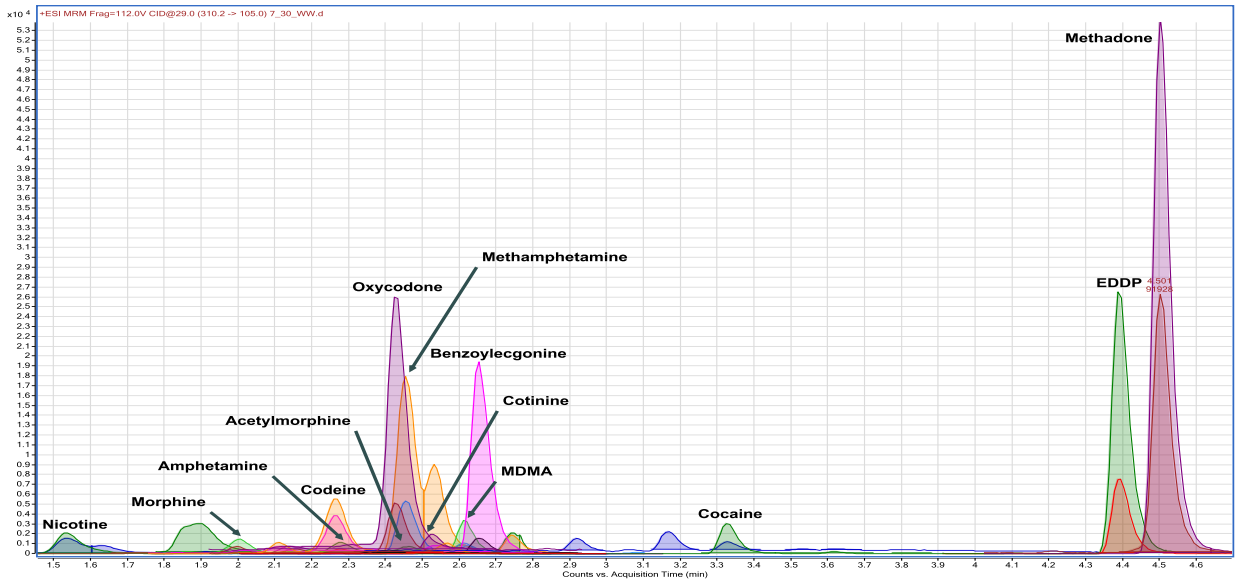

Figure 1: Chromatograph of a 100ng/ml standard.

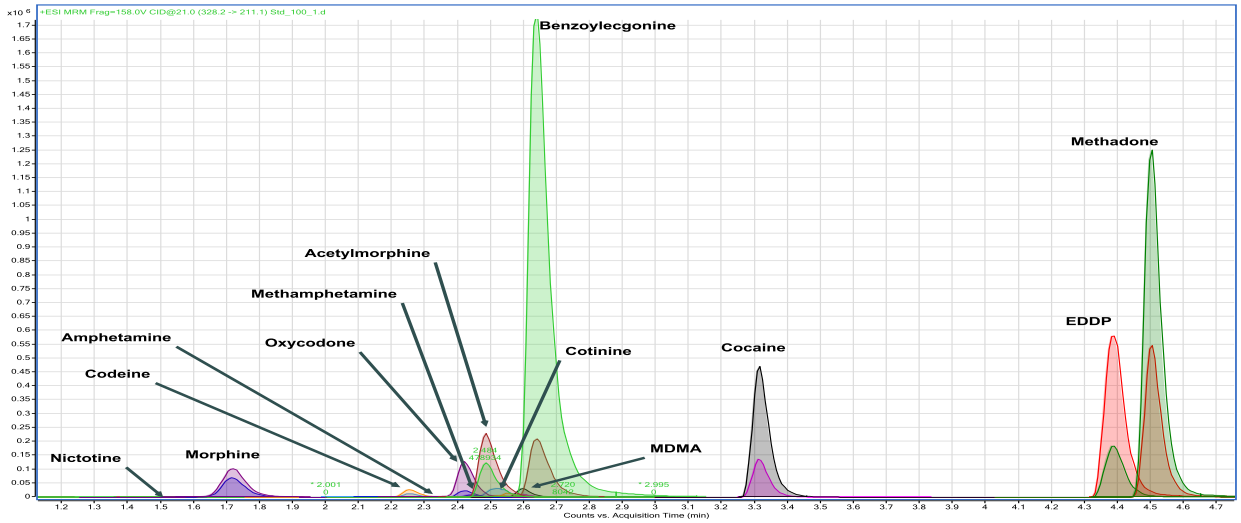

Figure 2: Chromatograph of a wastewater effluent sample from that was collected on July 30, 2011.

LC-MS/MS Analysis of Effluent Wastewater Samples

Samples of municipal wastewater effluent samples were collected over the course of six months in Fort Collins, CO. Samples were analyzed by a developed method using UHPLC-MS/MS. Samples were collected every day including weekends, and real-time data that was obtained can be seen in Table 3.

Concentration and mass loads of various illicit drugs showed a 7 day cycle which could be associated with weekend spikes. The consumption of certain drugs along with the metabolite associated with that particular drug showed similar trends, with corresponding spikes with the correlating weekend. Benzoylecgonine, the main metabolite of cocaine, showed the highest average mass load throughout the sampling time frame (57.76 mg/min). Cocaine was not as prevalent in wastewater effluent samples as benzoylecgonine but was still seen in the majority of wastewater samples with an average of 0.72 mg/min. The concentration of benzoylecgonine reached a mass load as high as 307.72 mg/min but was also as low as 0.17 mg/min throughout the sampling time frame, while cocaine was below the detection limit in a number of daily samples but reached a concentration as high as 30.27 mg/min during late October.

Nicotine, along with its major metabolite cotinine, was seen in every sample collected between July and December. Nicotine (17.58mg/min) had a higher average mass load than that of cotinine (3.75 mg/min). Both nicotine and cotinine followed a 7 day pattern through the months of July thru September, and each of them showed their highest mass loads during a week in the middle of October where nicotine's mass load reached 109.71 mg/min and cotinine's was at 38.82 mg/min.

The prevalence of methamphetamine was much higher than that of its metabolite amphetamine throughout the study. Both, methamphetamine and amphetamine, were below the detection level in various samples throughout the time frame. The highest mass load seen for methamphetamine was 53.33 mg/min and for amphetamine it was 4.10 mg/min. The concentration of methamphetamine during the study decreased over

the course of the six month time frame possibly indicating a decrease in availability or use.

The concentration and mass load of methadone was very consistent throughout the sampling time frame aside from a single week in October. The metabolite of methadone, EDDP, also followed a similar pattern and was consistent with the concentration found of methadone. The average mass load of methadone was 12.50 mg/min and EDDP was 0.45 mg/min. Methadone and EDDP were both found in every wastewater effluent sample that was collected between July and December.

The presence and concentration of morphine and its major metabolite acetylmorphine was very sporadic throughout the sampling time frame. Morphine showed various spikes that would occur not only on weekends but during the week. Acetylmorphine showed its largest concentration during the second week in November and showed no correlation with morphine concentration. The average mass load of morphine was 16.31 mg/min with the highest spike being 119.33 mg/min. The highest mass load for acetylmorphine was 11.17 mg/min. On several occasions both of these drugs were below the limit of detection.

Oxycodone and codeine, two common pharmaceuticals showed a consistent mass load throughout the sampling time frame aside from a week in the middle of October. The average mass load for codeine was 8.9 mg/min and oxycodone was 18.36 mg/min. The mass load of MDMA showed various spikes throughout the study with the highest mass load occurring in July at 6.36 mg/min. MDMA was not detectable in all effluent samples and was below the limit of detection in several samples although

the average mass load over the course of six months was 1.12 mg/min. Real- time average mass loads for each of the drugs of abuse along with their various metabolites can be seen in Table 3. Weekday and weekend averages were taken over the course of the six month study (weekends consisted of samples collected Saturday, Sunday, and Monday).

Various drugs of abuse along with certain pharmaceuticals have varying mass loads depending on the time of year samples were collected along with day of the week. A two week sampling time frame in late July leading into early August showed spikes on two uninterrupted weekends (Figure 3). The weekday mass loads of Benzoylecgonine (major metabolite of cocaine), MDMA, and Methamphetamine are much lower than during the weekend showing a prevalence for the use of these drugs on weekends as opposed to weekdays.

A 12-day period during the 6 month sampling time frame shows the presence of Codeine, Oxycodone, Methadone, and EDDP. The mass loads for each of these analytes shows a consistent presence without corresponding spikes during the weekend (Figure 4). These pharmaceuticals may be abused in certain instances but this shows the consistent use of these drugs as opposed to abuse.

Table 3: Real-time average mass loads (mg/min) of illicit drugs and their various metabolites in effluent wastewater of Fort Collins, CO during weekdays and weekends.

Weekends were classified as samples collected Saturday, Sunday, and Monday due to wastewater dwell time at the WWTP.

	July		August		September		October		November		December	
	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend
Nicotine	28.80	22.89	15.12	15.83	15.05	15.98	38.23	27.05	8.71	8.91	3.48	4.79
Morphine	51.74	13.62	14.14	6.19	19.84	4.08	23.04	13.84	20.06	1.59	7.68	21.53
Codeine	19.94	15.95	7.83	7.78	4.69	4.54	21.99	9.42	4.35	2.79	4.01	5.64
Benzoylecgonine	23.01	43.65	7.56	11.36	22.69	28.52	82.84	99.50	74.11	81.91	78.09	159.96
Cotinine	2.65	2.46	2.19	2.28	1.95	2.24	12.02	7.70	2.04	2.17	1.91	2.48
Amphetamine	0.06	0.14	0.50	0.34	0.19	0.18	0.22	0.26	0.26	0.21	0.26	0.28
Oxycodone	27.66	22.02	14.70	15.18	9.48	10.30	51.63	29.33	8.49	7.55	8.29	10.81
Methamphetamine	4.08	6.62	3.16	4.18	3.28	3.16	10.99	4.79	1.02	0.98	1.06	1.47
MDMA	0.33	2.08	0.75	1.45	1.21	1.05	1.37	1.60	0.70	0.73	0.80	1.54
Acetylmorphine	-	0.01	0.14	0.17	0.06	0.05	0.03	0.01	1.50	1.61	0.17	0.22
Cocaine	0.05	0.13	0.23	0.28	0.15	0.18	0.47	4.97	0.33	0.32	0.34	0.47
EDDP	13.57	11.98	9.05	8.74	5.94	7.70	38.62	20.39	7.43	6.37	5.74	7.62
Methadone	2.88	2.14	1.72	1.85	1.17	1.19	5.11	2.86	1.54	1.06	1.40	1.86

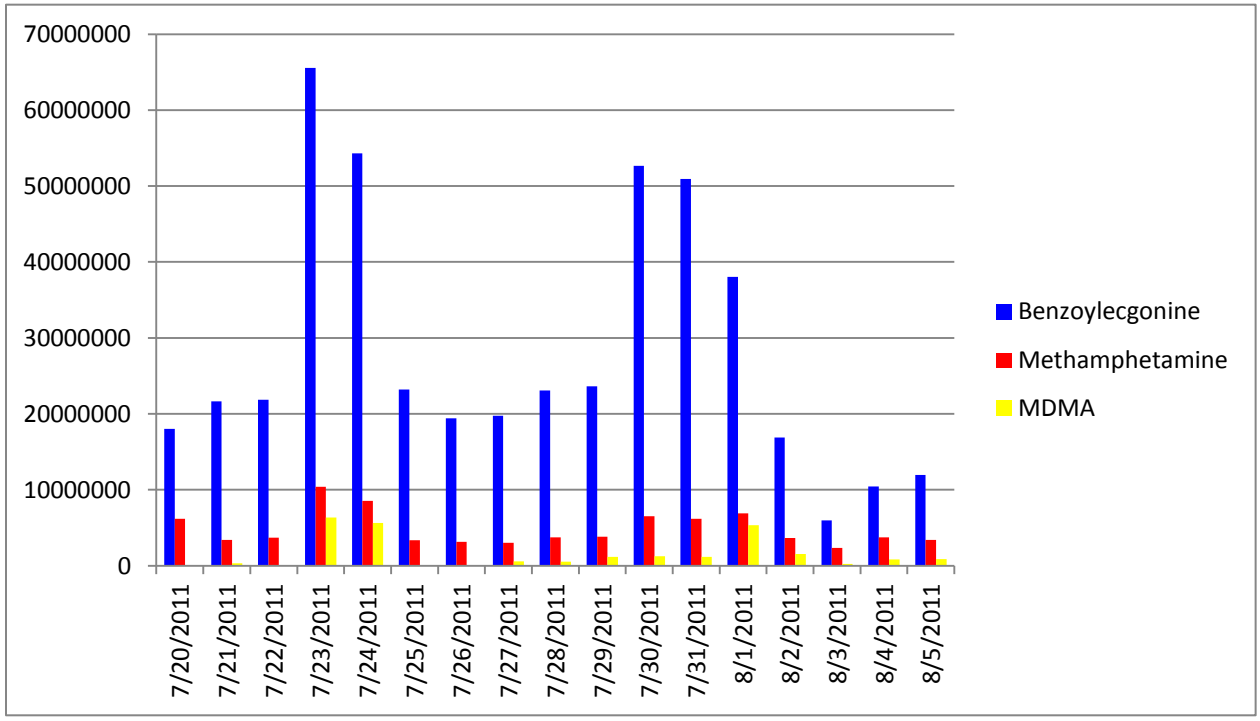

Figure 3. Two week sampling period showing spikes in mass loads (mg/min) in drugs of abuse on weekends as opposed to time during the week.

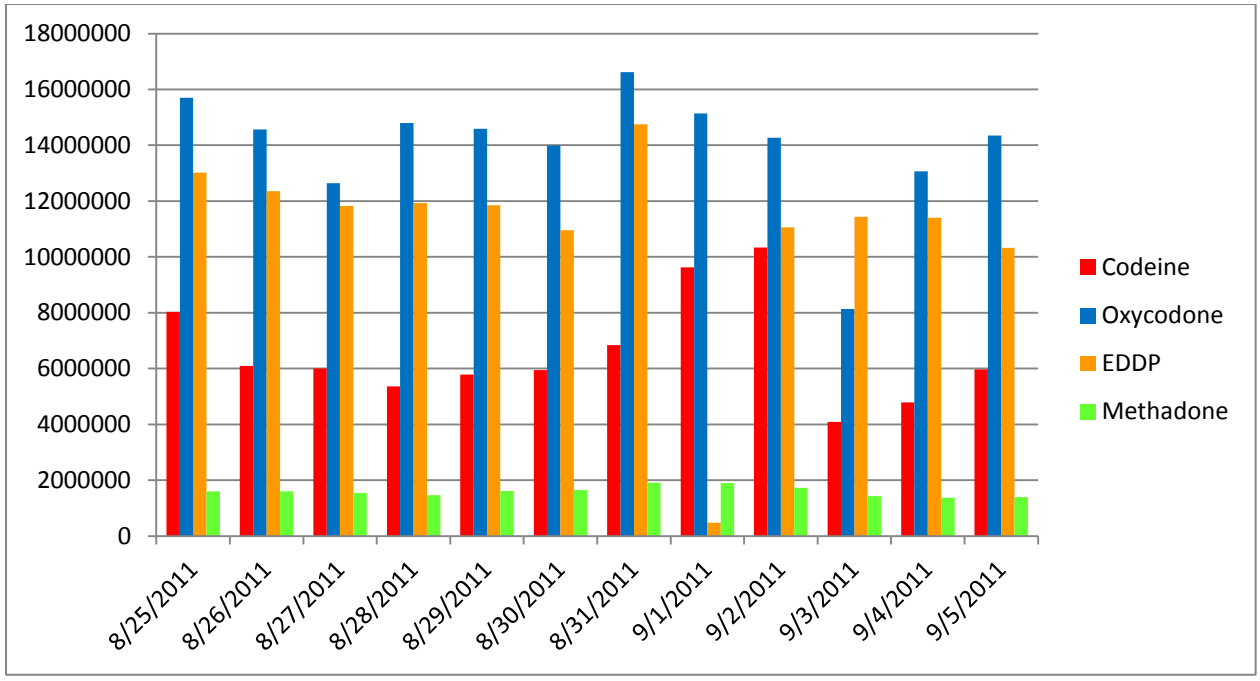

Figure 4. 12 day sampling period illustrating the presence of pharmaceuticals in effluent wastewater samples. Mass loads (mg/min) remained consistent with no weekend spikes and little variation.

Application of 24-hour Study

A number of 12 municipal wastewater effluent samples were collected every two hours over the course of 24 hours on May 7, 2012 in Fort Collins, CO. Two quality control samples were also analyzed with the 12 wastewater samples. The presence of each illicit drug as well as the various metabolites was seen in every sample during the course of 24 hours. The highest concentration found was that of Morphine (3.57 ng/mL) at 5 o'clock P.M. and the lowest concentration detected was Acetylmorphine (0.0039 ng/mL) at 1 o'clock A.M. Morphine and Benzoylecgonine contained the highest average concentration found throughout the sampling time, 1.56 ng/mL and 0.53 ng/mL, respectively. The highest loads found for each drug was seen in the hours between 5 P.M. and 11 P.M. with the exception of Amphetamine, Methamphetamine, Cocaine, and EDDP which had their highest load at 3 A.M., 3 A.M., 9 A.M., and 1 A.M., respectively. The total drug concentration was calculated for each time frame in which samples were collected (Figure 5). Morphine was not included in this calculation due to an abnormally large concentration of this drug found during each sampling time.

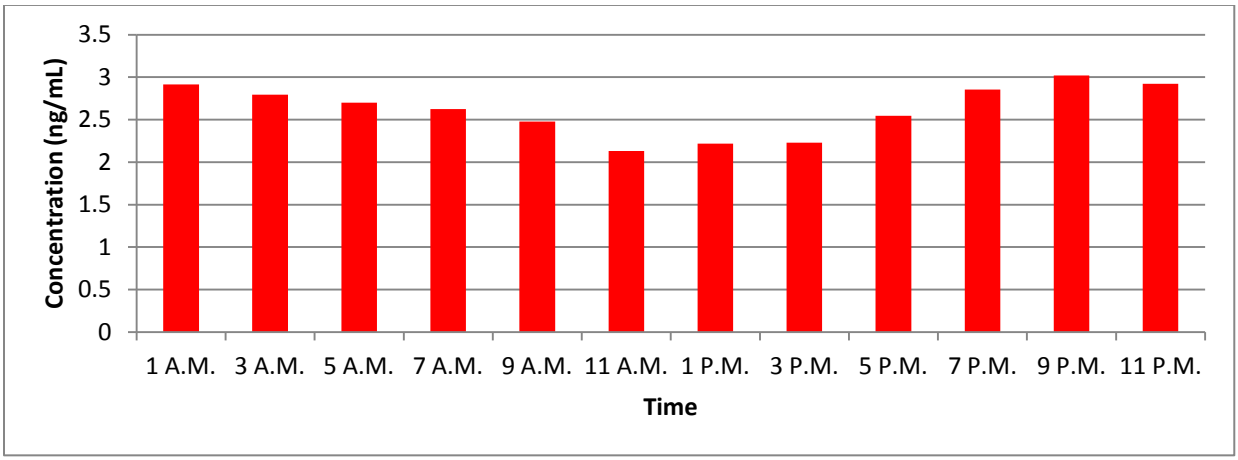

Figure 5. Total drug concentration found at each individual time frame. Morphine was not included in the total drug concentration.

4. Discussion

This study was conducted over the course of six consecutive months making it the longest study while measuring illicit drugs and metabolites in municipal wastewaters. The length of time over which this study was performed showed seasonal variation. Summer, fall, and winter months were included in able to study drug consumption patterns that arose due to changing weather and social patterns. The change from summer to fall also added a new demographic of college students into the local community. This study provided information on the varying drug consumption patterns with the new demographics' introduction into the area. The application of the developed method to effluent samples showed an increase in various drugs of abuse during weekends as opposed to weekdays over the course of the study.

Wastewater analysis is a useful tool in the estimation and monitoring of local drug consumption. These results indicate that illicit and prescription drug consumption is contributing to the contamination of aquatic environments since wastewater treatment plants are not effective in removing them prior to release in effluents. WWTP effluents are therefore a significant source for illicit drug and pharmaceutical loading into various receiving waters (7). The highest mass load levels that were reported corresponded to oxycodone and the cocaine-metabolite benzoylecgonine. The large amount of benzoylecgonine could not only be attributed to the abuse of cocaine but also be linked to the hydrolysis of cocaine as it sat in the WWTP. Benzoylecgonine was the only metabolite that had a higher mass loads on weekends as opposed to weekdays throughout the entire study. Contamination by illicit drug residues appears to be

widespread, and the major source can be attributed to consumers. Although many of these mass loads are low, the presence of drugs and pharmaceuticals in surface waters may lead to pharmacological interactions causing toxic effects to aquatic organisms (13).

This study was designed to measure commonly abused drugs and their primary metabolite as an indication of consumption or abuse within the Fort Collins community. It's important to take into consideration that certain metabolites may be consumed directly. However, it is not possible to distinguish between which drugs are illicit and which ones are prescribed throughout this study. The abuse of methamphetamine along with other pharmaceuticals that are metabolized into amphetamine, contribute to amphetamine's presence in wastewater. Morphine, like amphetamine, may not only come from the abuse of heroin but may also come from the therapeutic use of this drug (4). Certain aspects need to be taken into consideration when sampling wastewater: days of the week, seasons, and special events because each of these factors may contain unusual flow patterns and contaminant loadings (12).

Each of the drugs of abuse and their various metabolites that were looked at in this study were all detected at some point within the sampling time frame, with many of them seen on a day-to-day basis. Mass loads for various metabolites showed increases on weekends while others showed no change from samples obtained during the weekdays. Benzoylecgonine was the only analyte that showed an increased mass load during every weekend throughout the six months. The average mass load of cocaine was also higher during the weekends except during the month of November

where the weekday average mass loads were 0.33 mg/min and the weekend was 0.32 mg/min. MDMA followed a similar trend to cocaine, with higher average mass loads during the weekend as opposed to the weekdays except during the month of September. MDMA was not seen consistently throughout the sampling timeframe, however. The largest mass loads of MDMA were seen on a corresponding weekends during the end of July and beginning of August. Four out of the six months where samples were collected showed higher concentrations of nicotine and cotinine on weekends. There were various weekend spikes of methamphetamine in August and September, and during a week in October. During the course of the study codeine, along with oxycodone, showed relatively consistent mass load levels not dependent on weekend versus weekday. Each of these drugs is a prescribed pharmaceutical and each of them is not often abused.

The varying mass loads for certain metabolites showed changes associated with monthly climate and time of the year. The six month sampling time frame included month associated with summer, fall, and winter. The presence of methamphetamine was seen more often during warmer months (July, Semptember, August) as opposed to cooler months (November and December). During the months of November and December, the mass load for methamphetamine was lower than the previous months and did not show spikes corresponding to weekends. Every sample that was collected over the course of six months contained nicotine and cotinine. Warmer months had a higher average mass load of nicotine and cotinine than that of colder months. Benzoylecgonine showed a higher prevalence in wastewater samples during colder months as opposed to warmer months, unlike many of the other analytes. During the

month of July, the average weekday mass load of benzoylecgonine was 23.01 mg/min while in the month of December the average weekday mass load was 78.09 mg/min. The seasonal variation of many of the drugs found in effluent samples may depend on seasonal changes along with access to each of these analytes. The availability of many of these drugs may increase during the summer as opposed to months that have lower average temperatures.

During a week in October coinciding with midterms at the local university, a large spike in the mass load of every drug during the week including nicotine and cotinine was seen. The mass load seen during this week in October for benzoylecgonine was more than twice as high as any previous value up to that point. Methamphetamine showed a marked increase during this week in October reaching its highest mass load throughout the entire sampling time frame (53.33 mg/min). Methadone, EDDP, Oxycodone, and Codeine all showed spikes over three times as large as any mass load seen throughout the sampling time frame, during this week in Ocotober. Morphine and acetylmorphine were the only analytes that didn't show an irregular spike during the mid-terms week in October.

Every analyte was detected in every sample collected during the 24-hour study. The profiles for each of these analytes suggest that the mass loads that were found reflect real human excretion. A cyclical pattern, possibly coinciding with regular urination patterns, was seen over the course of 24 hours with the highest concentration of analytes occurring at 9 P.M. The lowest concentration of analytes was seen at 11 A.M. but the difference between the highest and lowest concentration was <1 ng/mL.

Morphine had the highest individual concentration of each of the analytes at 3.57 ng/mL and was not included within the average concentration of the individual time frame averages.

Morphine and acetylmorphine were not seen in every sample collected throughout the study. There were large spikes seen at various times over the course of six months in morphine which may be attributed to the disposal of this drug by pharmaceutical dispensaries, hospitals, and clinics. There were no large increases in the presence of acetylmorphine throughout the study and when acetylmorphine was seen, it was in small quantities. This would indicate that a large amount of the morphine that was seen was due to dumping as opposed to human excretion after it is metabolized throughout the body.

The drugs of abuse that were studied showed unique patterns based on the drugs as well as the weekend abuse, time of the year, and usage. The analysis of each of these analytes may be used to investigate patterns of consumption. This analysis can continue to be used not only in the community of Fort Collins but communities throughout the United States to monitor community drug usage and consumption. This analysis and study provide information showing that WWTP provide a significant source for illicit drug and pharmaceutical loadings into receiving water. These contributions of drugs and pharmaceuticals into surface waters may lead to toxic effects in aquatic organisms.

Using this study as a branching point into further research may help to provide insight into possible drinking water contaminants as well as how long these

drugs actually persist within the environment. This methodology could also be used to view the presence of illicit drugs in aquatic organisms' bodily fluids and organs. Particular knowledge on different aspects within the wastewater treatment process including time of residence of each drug, the amount of light exposure each drug sees, and the temperature conditions would help calculate drug consumption and usage as well (4).

5. Conclusion

Analytical methodology using solid-phase extraction and the use of LC-MS/MS showed the presence of varying amounts of different drugs of abuse along with their various metabolites to be found in municipal wastewater treatment effluent samples in Fort Collins, Colorado. Solid-phase extraction using Waters MCX columns proved to be an effective clean up procedure with spike recoveries of drugs ranging from 71% to 115% with limits of quantitation down to 5.9 ng/L. Possible analytical errors that may be associated with sample preparation or those that may result from matrix effects were compensated for by using deuterated internal standards. The collection of water samples that were analyzed showed measureable concentrations of all drugs in the wastewater samples with spikes on the weekends during the course of this six month observation. The other drugs and metabolites that were being analyzed stayed consistent in their concentrations throughout the various weeks of the study, with a slight increase of various drugs on certain weekends. Each of the drugs had a varying range depending on the time and day of the week. The drug with the largest range was Benzoylecgonine (0..43 mg/min – 307.72 mg/min). The approach of wastewater

analysis has proved to be a suitable method for assessing drug consumption of a population by providing real-time patterns of drug consumption.

References:

(1) UNODC. United Nations Office of Drugs and Crime World Drug Report 2011. 2011; United Nations: New York.

(2) Martinez Bueno, M. J., Ucles, S., Hernando, M.D., and Fernandez-Alba, A. R. 2011. Development of a solvent-free method for the simultaneous identification/quantification of drugs of abuse and their metabolites in environemental water by LC-MS/MS. *Talanta;* 85: 157-66.

(3) Gonzalex-Marino, I., Quintana, J. B., Rodrigues, I., Gonzalez-Diez, M., and Cela, R. 2011. Screening and selective quantification of illicit drugs in wastewater by mixed-mode solid-phase extraction and quadrupole-time-of-flight liquid chromatography-mass spectrometry. *Analytical Chemistry;* 84: 1708-17.

(4) Nuijs, A.L.N., Castiglioni, S., Tarcomnicu, I., Postigo, C., de Alda, M.L., Neels, H., Zuccato, E., Barcelo, D., and Covaci, A. 2011. Illicit drug consumption estimations derived from wastewater analysis: A critical review. *Science of the Total Environment*; 409: 3564-77.

(5) Castiglioni, S., Bagnati, R., Melis, M., Panawennage, D., Chiarelli, P., Fanelli, R., and Zuccato, E. 2011. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. *Water Research*; 45: 5141-50.

(6) Gracia-Lor, E., Sancho, J.V., and Hernandez, F. 2010. Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry. *Journal of Chromotography A;* 1217: 622-32.

(7) Bartelt-Hunt, S. L., Snow, D. D., Damon, T., Shockley, J., and Hoagland, K. 2009. The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska. *Environmental Pollution;* 157: 786-91.

(8) Pedrouzo, M., Borrull, F., Pocurull, E., Marce, R. M. 2011. Drugs of abuse and their metabolites in waste and surface waters by liquid chromatography-tandem mass spectrometry. *J. Sep. Sci.;* 34: 1091-1101.

(9) Banta-Green, C. J., Fields, J. A., Chiaia, A. C., Sudakin, D. L., Powers, L., and de Montigny, L. 2009. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater. *Addiction;* 104: 1874-80.

(10) Fent, K., Weston, A.A., and Caminada, D. 2006. Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology;* 76:122-59.

(11) Phillips, P. J., Smith, S. G., Kolpin, D. W., Zaugg, S. D., Buxton, H. T., Furlong, E. T., Esposito, K., and Stinson, B. 2010. Pharmaceutical formulation facilities

as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. *Environ. Sci. Technol.;* 44: 4910-16.

(12) Gerrity, D., Trenholm, R. A., and Snyder, S. A. 2011. Temporal variability of pharmaceuticals and illicit drugs in wastewater and the effects of a major sporting event. *Water Research;* 45: 5399-411.

(13) Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., and Fanelli, R. 2008. Illicit drugs, a novel group of environmental contaminants. *Water Research;* 42: 961-68.

(14) Bijlsma, L., Sancho, J. V., Pitarch, E., Ibanez, M., and Hernandez, F. 2009. Simultaneous ultra high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. *J. of Chromatography A;* 1216: 3078-89.